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CHARACTERIZATION OF BACTERIAL GENERA, SPECIES, AND VARIANTS BY INFRARED ANALYSIS AS RELATED TO THE STUDY OF AIRBORNE BACTERIA

By

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TABLE OF CONTENTS

This report contains 46 pages.

LIST OF FIGURES

LIST OF FIGURES (Continued)

Page

LIST OF TABLES

SUMMARY

The potassium bromide disc technique for the quantitative infrared spectrophotometric analysis of whole bacterial cells has been found satisfactory and, in addition to the potassium bromide's inherently excellent qualities as a suspending matrix, has been found expedient to the particular circumstances under which this exploratory work was carried out. The incorporation of an internal standard in the basic potassium bromide matrix was found to be very useful as a wavelength marker, as a baseline check on each spectrum as run, and as an indication of disc quality.

The chromogenic variants of Serratia marcescens ATCC 274 are not distinguishable by the spectral results obtained. Hence, attempts to monitor the standard test culture and seek some correlation with aerial viability and infrared spectral characteristics do not appear presently practical under the conditions of instrumentation outlined. There may be some basis at the genera level and species level for relating aerial viability and spectral groupings. These groupings may not strictly follow the classification nomenclature.

SIGNIFICANT ACCOMPLISHMENTS

Techniques of quantitative chemistry were applied to the problem of differentiating bacterial variants by the use of infrared spectrophotometry. Results indicate that these techniques produce a dependable accuracy of measurement and reproducibility. The incorporation of an internal standard provides, in addition to a wavelength marker, a quantitative baseline reference for the bacterial spectrum. These procedures afford a duplicable method of quantitating the spectral characteristics of bacteria and a more reliable method of comparative spectroscopy of whole bacterial cells.

Comparative analysis of bacterial genera's spectra indicates a possible relationship with the viability of the airborne bacterium, but the absence of more cumulative data prevents drawing any conclusions. The inability to apply the infrared spectrophotometric method to the differentiation of bacterial variants used as aerial test organisms defines further the results of other workers of the limiting instrumental sensitivity and the necessity of employing cell fractionation to determine differences existing between closely related organisms.

I. PREFACE

The purpose of this study was to make an exploratory investigation of the utilization of infrared spectrophotometry for the characterization of a selected variant bacterium which is employed as a standard test organism in studies of the atmospheric factors related to bacterial viability in the airborne state. Continuing cultural stability of the test organism used in these aerial studies is of prime importance to the collection of reliable data. Whether or not this stability is being maintained in the test culture can be ascertained by exposure of the test organism in the airborne state to a standard atmospheric condition. However, this assay procedure is highly consumptive of time and materials. The published reports of the successful application of infrared spectroscopy to the identification of bacteria suggested that infrared analysis might offer a direct and simplified monitoring procedure in the maintenance of the standard test culture.

In addition, the differential survival of genera and species of bacteria in the airborne state suggested the application of infrared spectroscopy as a means of demonstrating a possible spectral correlation with aerial viability. If peculiar spectral expressions were found in association with particular aerial viabilities, a presumptive basis would be established for further studies which might elucidate some of the responsive fundamental biological mechanisms operating in the airborne bacterium.

II. HISTORICAL BACKGROUND

Since the first paper[1] reporting the use of infrared spectrophotometry in the whole cell identification of bacteria, there have been a number of published papers dealing with this subject. The most inclusive and extensive studies reported have been those of Thomas and Greenstreet,[2] Riddle, et al.,[3] and Greenstreet and Norris.[4] There appears to be a general agreement that infrared spectrophotometry forms a reliable basis for bacterial identification and classification. Genera are easily distinguished[4] but the difficulty of differentiation increases at the species level,[4] with certain species being inseparable at the present state of technique development.[3]

The difficulty of differentiating strains of bacteria becomes very great, the differences being less than errors inherent in the technique employed.[4] However, some of the spectral differences accounting for poor reproducibility among some species have been attributed to strain differences.[3] In the infrared study of resistant strains of bacteria Kull, et al.,[5] noted qualitative spectral differences only between certain antibiotic resistant and sensitive strains. These deviations could not be quantitatively related to the degree of resistance. The conclusion was that the spectral pattern of whole bacterial cells did not present an unequivocal identification of bacterial species unless supported by corroborative evidence.

III. EQUIPMENT

A. Airborne Studies

The aerosol test chamber employed for the determination of aerial viability is a 4-foot cube constructed of tempered Masonite. The bacterial aerosol, composed of single cell particulates, is introduced into the inlet pipe leading to the main chamber and is mixed with the main air stream. It is then uniformly distributed throughout the chamber by an air diffuser located in the center of the top of the chamber and is removed from the chamber through an exit pipe in the center of the bottom. The entire system is located in a room of controlled atmosphere.

The system is operated dynamically at 60 cubic feet per minute air flow with zero pressure differential. Static test conditions are established by diverting the aerosol source to the bypass, shutting off the air-blower system, and sealing off the inlet and outlet pipes. This arrangement makes sampling conditions possible under dynamic operation of the system at approximately one volume change per minute, and also makes possible the determination of the biological die-away under static conditions.

During dynamic operation of the chamber, air samples are taken from the inlet (n_o) to the chamber and from the outlet (n_1) by means of the critical orifice liquid impinger samplers. The aersol detention time or aerial exposure (t) under these conditions is 5 minutes. The death rate of airborne bacteria is expressed in terms of the biologic die-away constant, k:

$$k = \frac{\log n_o - \log n_1}{t}$$

The critical orifice liquid impinger sampler was operated at 1.0 liter per minute for a sampling period of 10 minutes. Each sampler contained 200 ml of a solution of 0.2 percent gelatin (Pharmagel A)† buffered with 0.08 gram anhydrous sodium mono-H orthophosphate (Na_2HPO_4) per gram of gelatin and enriched with 1.6 per cent of brain heart infusion (Difco). A bacterial inoculating loopful of Dow AF antifoaming agent was added to each sampler. Aliquots of the sampler fluid were plated in triplicate in tryptone glucose extract agar to which was added 2.5 grams of sodium mono-H orthophosphate (anhydrous) and 5 grams of sodium chloride per liter.

Under static chamber conditions, the die-away constant, k, was determined from the one-minute sequential exposure of settling plates placed in exposure ports in the chamber bottom. These settling plates were filled with nutrient agar (Difco). The slope (k) of the line resulting from a plot of numbers of viable cells (colonies) versus time on semilogarithmic paper is the biologic die-away under static conditions.

All culture plates from samplers, dynamic and static, were incubated at 30° C and counted after 18 hours incubation. The standard atmospheric test condition for all determinations of aerial viability was 68° F and 65-per-cent relative humidity.

Detailed descriptions have been published previously of the operation and performance of the aerosol test chamber[6] and the critical orifice liquid impinger samplers.[7]

†Milligan and Higgins Corp., 222 - 224 Front Street, New York 38, N. Y.

B. Infrared Analysis

1. Instrumental Analysis

All spectra were obtained with the Perkin-Elmer Model 21, double-beam recording infrared spectrophotometer located in a room of constant temperature and relative humidity.

On each day of use, and before the running of an analysis, the instrument was permitted sufficient time to warm up prior to making setting adjustments. Internal instrument balance was attained with both beams closed and the servo-mechanism indicating 50-per-cent transmission. The amplifier was adjusted so that the indicator position was stable or showed a slight upward (toward 100 per cent) drift. With the wavelength indicator at 5 microns, the zero transmission setting was made by opening the reference beam and permitting the response to become stabilized before manually resetting the indicator to zero, if required. The zero setting was then induced to an upward drift of 0.5 to 1 per cent by the amplifier control. Opening the sample beam brought the response indicator to approximately 100-per-cent transmission, the exact transmission value of 100 per cent being made by the suitable control. The procedure was repeated again as a check on the instrument performance to this point.

In order to check the wavelength calibration of the instrument, the spectrum of the polystyrene standard film was obtained each day prior to an analysis of any bacterial culture. The particular instrument used in these studies showed a constant deviation of wavelength setting of 0.05 micron from the polystyrene film standard. This deviation was compensated for by advancing the chart-drum. Also prior to starting of spectrum recording, the chart-drum was manually set in a positive mechanical forward position to avoid a slight lag caused by the gear-drive mechanism of the chart-drum.

The standard instrument settings for all spectra obtained were: Resolution 927, Response 2:2, Gain 5, Speed 5 (1.5 minutes/micron), Suppression 6, Scale 2 inches/micron, and the Energy Source 0.38 amperes. Spectra were recorded on Perkin-Elmer transmission charts (No. C 21-6301). For report reproduction and analysis, transposition of the spectra to arithmetical paper[†] was done by plotting transmission values at wavelength intervals not exceeding 0.1 micron, the entire transmission spectrum being related to setting the transmission of the internal standard at 60 per cent.

2. Potassium Bromide Die and Press

The Hilger Die[††] was used in the preparation of all potassium bromide discs. This die is evacuable and removal of the pellet from the die is facilitated by ease of disassembly of the die components. Dies 13 millimeters in diameter are produced.

The standard potassium bromide-bacteria mixture was removed from the storage oven (215° F) and placed in the die-well. The mixture was evenly distributed in the well with a chrome spatula; the plunger was then inserted and rotated in one direction with slight pressure to condense the material. The plunger was removed, polished-face disc component inserted and the remaining components of the die assembled in place.

The readied die was attached to a vacuum source (Hyvac laboratory pump) for a period of 5 minutes to remove entrapped air. Following evacuation of the die, a total pressure of 18,000 pounds was applied to the die for a period of

[†] Codex Book Co., Norwood, Mass. -- Cat. No. 31190

[††] Hilger and Watts, Ltd., Hilger Division, 98 St. Pancras Way, Camden Road, London, N. W. 1, England.

10 minutes. Initially, the Carver manually operated laboratory press was used for this purpose. The die was recently adapted for use in the Dietert automatically operated hydraulic press. Both presses were found to be satisfactory, the latter being used with greater facility.

Upon removal from the press, the die was disassembled, and the finished disc removed and stored in screw-cap vials placed in the 215° F oven until needed for spectral analysis. Care was taken in the extrusion of the finished disc from the die to avoid peripheral fractures of the potassium bromide-bacterial disc. These fractures were avoided by slightly warming the inner die barrel component and extruding the disc by a manually operated screw-type press (Hilger). A disc was adjudged optically satisfactory if distant objects were visible when viewed through it. Discs of this quality were found to be quite stable under storage conditions of 215° F or when left at room temperature in the screw-cap vials even without the addition of desiccating agents.

IV. MATERIALS AND METHODS

A. Bacteriological

1. Airborne Studies

Cultivation of all organisms preparatory to the determination of the aerial viability was in 60 ml of 0.3-per-cent-beef-extract broth contained in a 125-ml Erlenmyer flask and incubated at 30° C. Each culture was serially transferred at intervals of 40 to 45 hours for the routine maintenance of the liquid culture. Initiation of the liquid culture of all organisms following transfer from other media or storage conditions was followed by at least three transfers through the 0.3-per-cent-beef-extract broth before testing of aerial viability. Stock cultures were maintained on refrigerated nutrient agar slants at 5° C.

Cultures selected for aerial viability tests were 40 to 45 hours old. Previous work with Serratia marcescens ATCC 274 and its chromogenic variants has shown this age culture to be in the late phase of negative growth acceleration or in the early phase of the stationary period of the growth cycle. Cultures of this age have proved to be the most stable for routine use, the variations which might be expected from a younger, more rapidly proliferating culture being absent. Other bacterial genera selected for aerial viability studies were also tested at this same age of cultural growth.

The airborne organisms from the dynamically operating chamber system were collected into a solution of 0.2 per cent gelatin (Pharmagel A - Pharmagel Corporation) buffered with 0.08 gram anhydrous sodium mono-H orthophosphate per gram of gelatin and enriched with 1.6 per cent of brain heart infusion (Difco). A loopful of Dow AF antifoaming agent was added to each sampler. This collection

medium has been shown[7] to be highly effective in the recovery of viable bacterial cells from the airborne state. Immediately following collection of the aerosol sample, aliquots of the sampler fluid were plated in triplicate. The plating nutrient medium was tryptone glucose extract agar to which were added 2.5 grams of anhydrous sodium mono-H orthophosphate and 5 grams of sodium chloride per liter.

Static settling samples were obtained by gravitational deposition of the bacterial aerosol on the solidified surface of nutrient agar contained in Petri dishes placed at floor level within the aerosol chamber. The addition of an enrichment medium to the nutrient agar settling plates has not been shown[8] to increase the recovery of the settled cells. Likewise, additional enrichment of the nutrient medium used in plating aliquots from the liquid impinger samples does not increase the numbers of viable cells demonstrated.[7]

Culture plates from samplers, dynamic and static, were incubated at 30° C for a period of 18 hours before counting the developed colonies for the determination of bacterial counts.

2. Chromogenic Variants of Serratia marcescens ATCC 274

The chromogenic variants of this bacterial strain were differentiated by the use of the synthetic medium reported by Bunting.[9] This medium is composed of glycerol, 5 grams; ammonium citrate, 5 grams; potassium mono-H orthophosphate ($K_2HPO_4 \cdot 3\ H_2O$), 10 grams; magnesium sulfate , 0.5 gram; sodium chloride, 0.5 gram; ferric ammonium citrate, 0.05 gram; distilled water, one liter; agar, 1.5 per cent. The hydrogen-ion concentration is 6.9.

A small "bead" of bacterial growth was removed from a nutrient agar slant culture and diluted (10^6) in sterile water. Two drops of this bacterial

suspension streaked over the surface of the solidified Bunting medium produced 100 to 200 colonies per plate. Multiple plates were made in order to secure the nonpigmented variant which occurs with considerably less frequency than the blood-red, red, and pink pigmented variants. The inoculated plates were stored in a darkroom at 20° C for a period of 72 hours, the developed colonies then being approximately one millimeter in diameter. Typically appearing colonies of blood-red, red, and pink pigmentation, and white colonies were transferred to 0.3-per-cent-beef-extract broth and incubated at 30° C for 45 to 48 hours. These liquid cultures were diluted in the same manner as was the growth from the nutrient agar slant culture. The liquid cultures were streaked on Bunting's solid medium. The plates were incubated at 20° C for 72 hours and checked for completeness of separation. The variant types selected from these plates were transferred to screw-cap tubes of nutrient agar and incubated for 40 to 45 hours at 30° C. These cultures were then stored at 5° C and comprised the stock of variants from which inoculations could be made preparatory to the testing of aerial viability. This procedure for the isolation of variant types has been successful in the selection of relative stable types.

3. Bacterial Culture for Infrared Analysis

The culture medium[†] employed for the growth of bacterial cultures for subsequent infrared analysis was composed of tryptose, 20 grams; sodium chloride, 5 grams; thiamine, one milliliter of a 0.01-per-cent solution; ferrous sulfate ($FeSO_4 \cdot 7\ H_2O$), one milliliter of a one-per-cent solution; agar, 25 grams; distilled water, 1000 ml. The pH of the completed medium was 6.8 ± 0.1. The medium was added to square-shaped quart-size milk bottles in an amount sufficient to produce a maximum surface area when the bottle was laid on a side. The bottles containing

[†]Composition obtained by personal communication: Mr. Bernard A. Kenner, Taft Sanitary Engineering Ctr., Cincinnati, Ohio.

the medium were plugged with cotton, sterilized, and, upon removal from the autoclave, laid on a side for solidification of the agar. Shelf storage-life of the prepared bottles was extended by capping of the bottles with sterile aluminum foil. Immediately prior to the start of the determination of the aerial viability of a test organism in the aerosol system, a transfer was made from the 45- to 48-hour-old, 0.3-per-cent-beef-extract broth culture by placing two drops of culture onto the surface of each of two bottles containing the infrared test culture medium. The drops of culture were distributed over the surface of the medium by streaking with a sterile glass rod having a 90° bend approximately 15 millimeters from the end. The inoculated bottles were transferred to a 30° C incubator and positioned at a slight angle to prevent flooding of the medium surface by the small amount of collected excess water.

The cultures, with given exceptions, were grown for 18 hours. The bacterial growth was harvested with the angled sterile glass rod by drawing the rod with very slight pressure over the medium surface in a manner not to disrupt the agar surface. The harvested culture from two bottles was pooled in a small glass vial which was stored in a drying oven at 210° F until completely dry.

Following the drying process, the culture was transferred to a small mullite mortar where it was ground to a powder. This powder was transferred to a small screw-cap vial, labeled, and stored in a vacuum desiccator containing silica gel until required for use.

B. Preparations with Potassium Bromide

1. Potassium Thiocyanate Internal Standard

A stock aqueous solution was made volumetrically by adding 0.6 gram of potassium thiocyanate (reagent grade) in 1000 ml of distilled water. To 24 grams

of optical grade potassium bromide, sufficient distilled water was added to effect complete solution of the salt. Ten milliliters of the stock potassium thiocyanate were added to the solution of potassium bromide and the combined solutions placed in the 215° F oven for evaporation.

The resulting crystalline mass was removed in small quantities to a mullite mortar and thoroughly ground. After thorough mixing of the recombined ground material, small quantities were ground in a Wig-L-Bug amalgamator† with two 1/8-inch stainless steel balls for 10 minutes per quantity of material. The material was again recombined, thoroughly mixed, and stored in the 215° F oven. This mixture in the amount of 200.0 milligrams weighed on an analytical balance formed the basic substrate of the pressed pellets. Pellets of greater weight were made by adding 100 milligrams of pure potassium bromide to the 200-milligram basic mixture. Disc pellets in the weight range of 200 to 300 milligrams were easier to manufacture than those of weight in the range of 100 to 200 milligrams.

2. Potassium Bromide - Bacterial Combination

The 300.0-milligram (potassium bromide - potassium thiocyanate, 200.0 mgm; potassium bromide, 100.0 mgm) weight of substrate was found to be the optimum amount to use. To the 300.0 milligrams of substrate 1.00 milligram of dried ground bacteria weighed on a microgram balance was added. This mixture was placed in a small mullite mortar and one milliliter of diethyl ether added. This semi-solution was ground to dryness followed by the addition of another milliliter of diethyl ether and grinding to dryness. This finely powdered dry material was transferred to a small screw-cap vial and stored in the 215° F oven for complete evaporation of the diethyl ether; it remained under this storage condition until pressed into the disc pellet form.

†Crescent Dental Mfg. Co., Chicago, Ill.

V. RESULTS

A. Airborne Studies

A number of determinations of the aerial viability of the species of Escherichia and of Serratia were made in order to obtain a firm k value for each organism. The results of these determinations are reported in Table I. Certain of the k values are of particular interest in that a significant difference is shown between the values for the viability under dynamic conditions and those obtained under static conditions.

TABLE I

AERIAL VIABILITY OF SELECTED SPECIES OF THE GENERA SERRATIA AND ESCHERICHIA

Genus	Species	ATCC No.	k	
			Dynamic	Static
Serratia	marcescens	274	0.042	0.046
	plymuthicum	183	0.069	0.055
	indica	4003	0.055	0.053
	anolium	6063	0.046	0.068
	urinae	11111	0.056	0.066
Escherichia	coli	10536	0.098	0.091
	freundi	8454	0.049	0.120
	intermedium	6750	0.040	0.064

Similar aerial studies on the chromogenic variants of Serratia marcescens ATCC 274 are reported in Table II. Not all determinations of k for these variants are reported in this table, only those showing particular values for both dynamic and static conditions of k determination. The standard test organism (initially selected on the basis of chromogenicity: red-variant)

TABLE II

AERIAL VIABILITY OF Serratia marcescens
ATCC 274 AND ITS ISOLATED CHROMOGENIC VARIANTS

Organism	k		Distribution of Color Variants			
	Dynamic	Static	BR (%)	R (%)	P (%)	W (%)
Serratia marcescens ATCC 274	0.042	0.046	93	5	2	0
Serratia marcescens BR-variant	0.056	0.066	99	1	0	0
Serratia marcescens R-variant (12-56)	0.049	0.043	48	17	23	12
Serratia marcescens R-variant (6-57)	0.009	0.083	95	5	0	0
Serratia marcescens P-variant (6-57)	0.054	0.055	0	<1	97.5	2.2
Serratia marcescens W-variant (6-57)	0.074	0.057	0	0	<1	99

responds to the standard conditions of atmosphere to produce dynamic and static k of 0.035-0.040. Deterioration of the standard test culture is first evidenced by a larger k under static conditions (Table II, R-variant/6-57), aerial viability alteration being demonstrated firstly by the solid surface sampler. This same variant also exhibits the extent of k fluctuation under dynamic conditions, a fluctuation which frequently accompanies the steady but high k values under static conditions.

The R-variant results in the most stable culture employed in the aerial viability studies. The loss of stability of this variant culture as a standard test organism (Table II, R-variant, 12-56; R-variant, 6-57) has been found to

be associated in as yet undetermined relationship with the ratio of other chromogenic variants present in the culture. The progressive shift of the ratio of color variants toward a predominance of the BR-variant type and the emergence of a wide morphological and dimensional variation of cell forms usually is accompanied by a deterioration of the k values under the standard atmospheric conditions.[8] Although the incidence of the W-variant is low in the undifferentiated Strain 274 of Serratia marcescens and disappears early in the progressive shift of the color variants, the k values were nonetheless obtained for this variant.

B. Infrared Studies

1. Potassium Bromide - Potassium Thiocyanate Disc Technique

To determine the relative accuracy which could be obtained with the potassium bromide method with an incorporated internal standard, several weighed amounts of bacteria(Serratia marcescens, ATCC 274) were incorporated in different discs of potassium bromide of the same weight. The results are shown in Figure 1, transcribed directly from infrared chart as recorded. Transmission values at selected wavelengths were plotted against bacterial concentration on semilogarithmic paper to determine the relative agreement with the Lambert Absorption Law (Figures 2 to 6). The actual deviations from the theoretical absorption values are tabulated in Table III. The deviations of the various bacterial concentrations show a standard deviation in the range of from 1 to 2 per cent. The potassium thiocyanate internal standard from six determinations shows a per cent transmission of 60.6 per cent at 4.85 microns wavelength with a standard deviation of 1.75 per cent. In comparing spectra of the various organisms examined, an error of 2 per cent was assigned. Selection of this largest variation from the above data enabled spectral comparisons to be made with a little more confidence in eliminating spurious similarities.

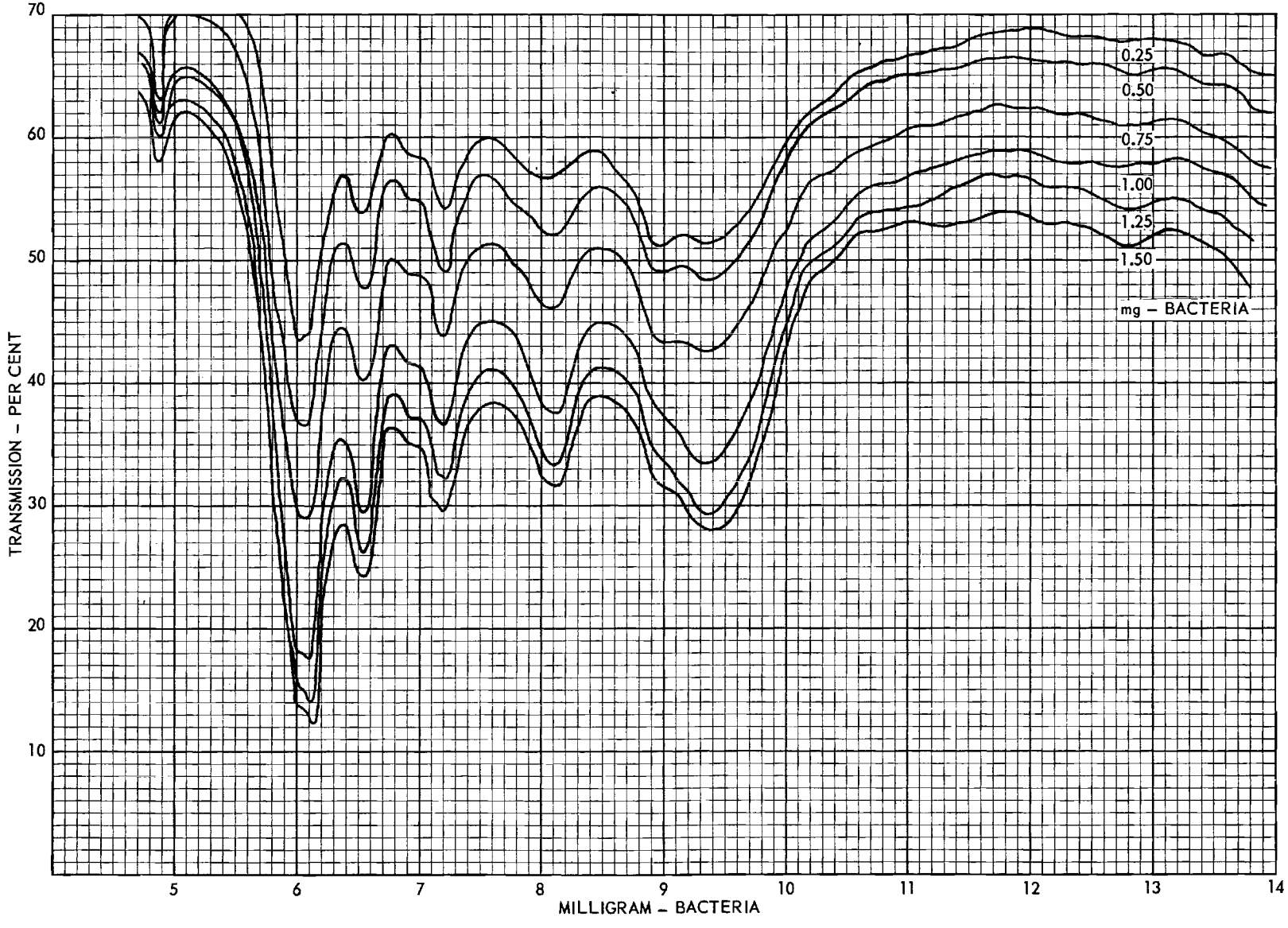

Figure 1. Per Cent Transmission Values of Different Concentrations of Serratia marcescens, ATCC 274.

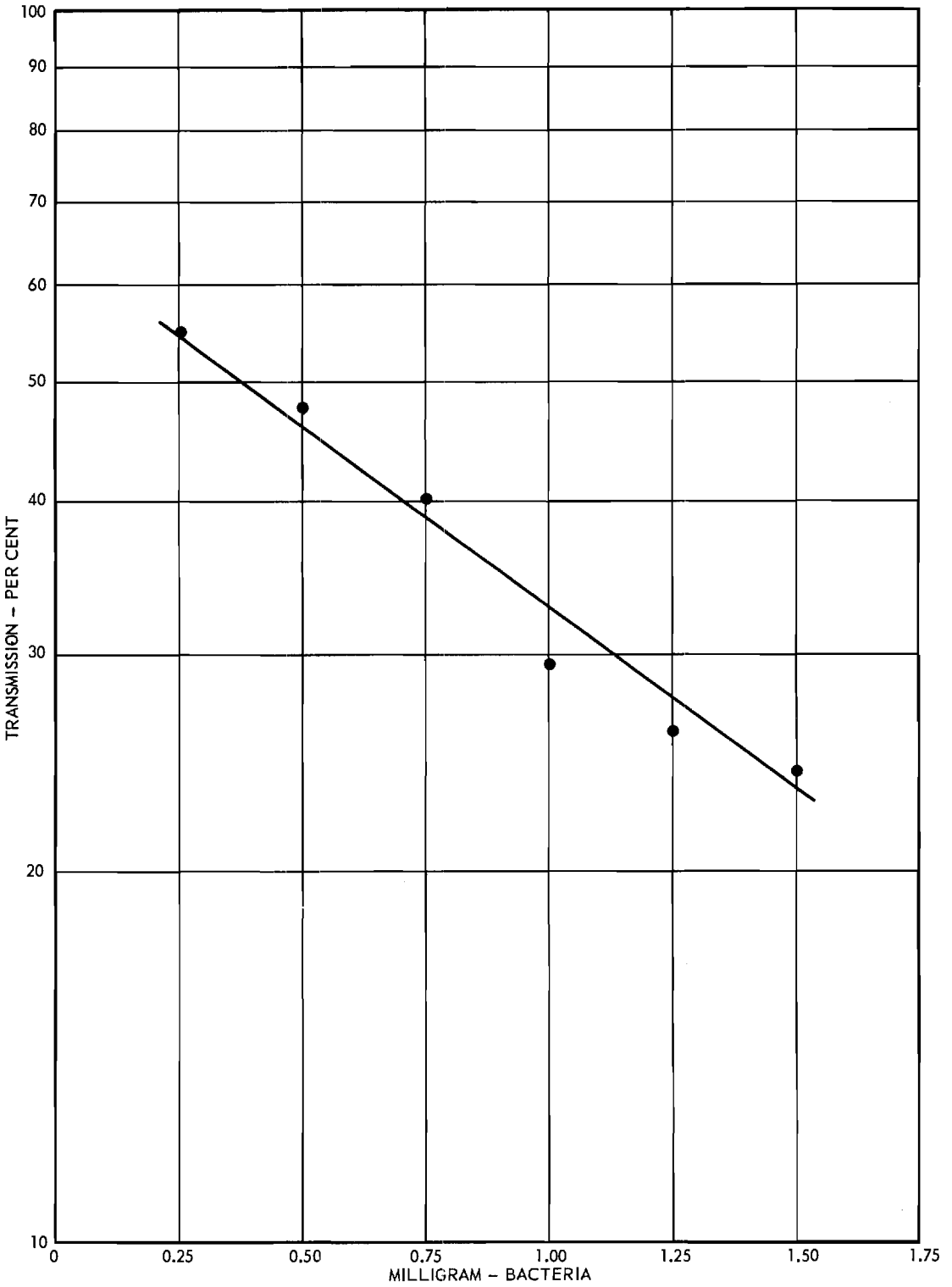

Figure 2. Per Cent Transmission Values of Different Concentrations of Serratia marcescens, ATCC 274, at 6.53 Microns Wavelength.

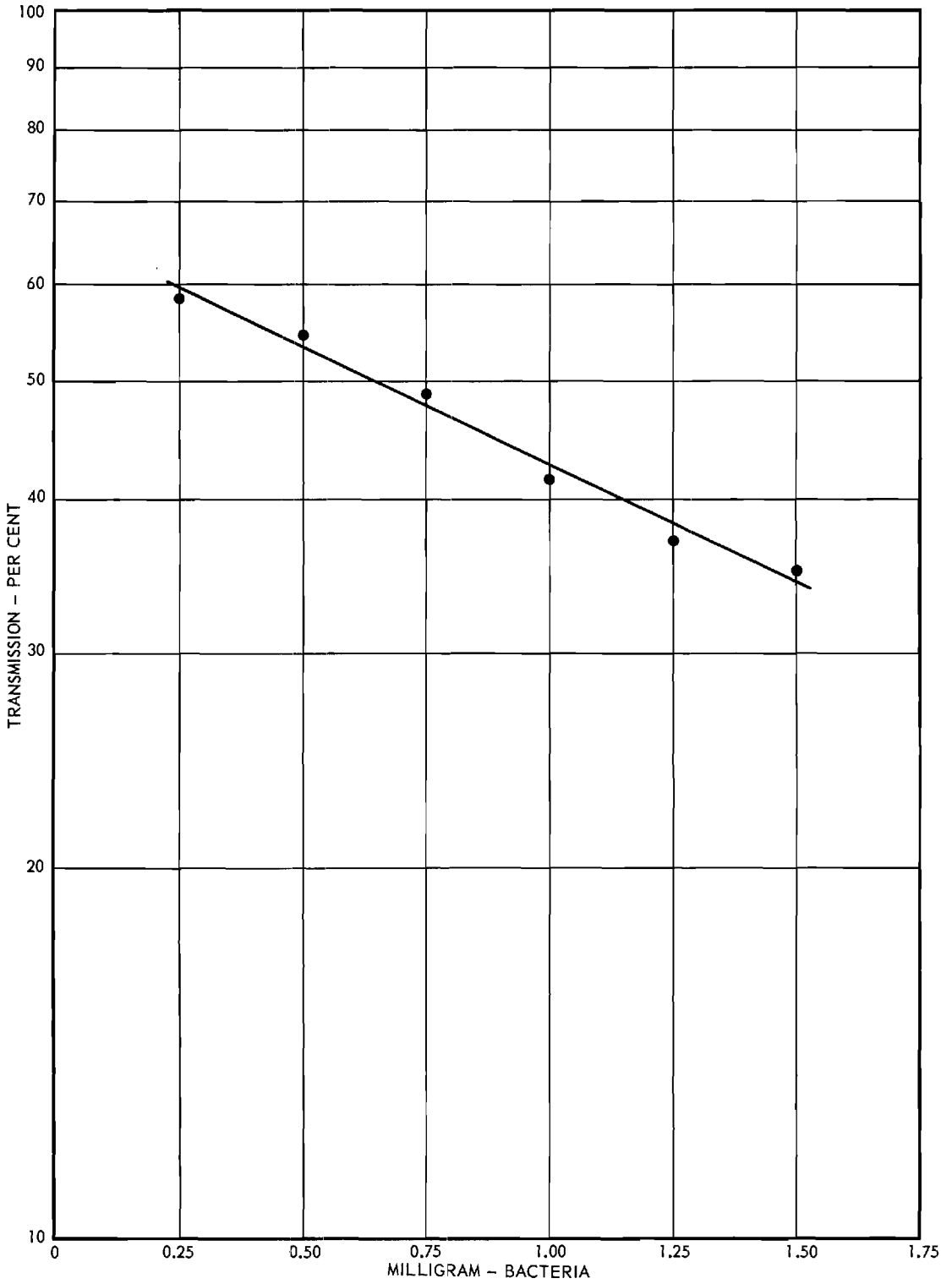

Figure 3. Per Cent Transmission Values of Different Concentrations of Serratia marcescens, ATCC 274, at 6.9 Microns Wavelength.

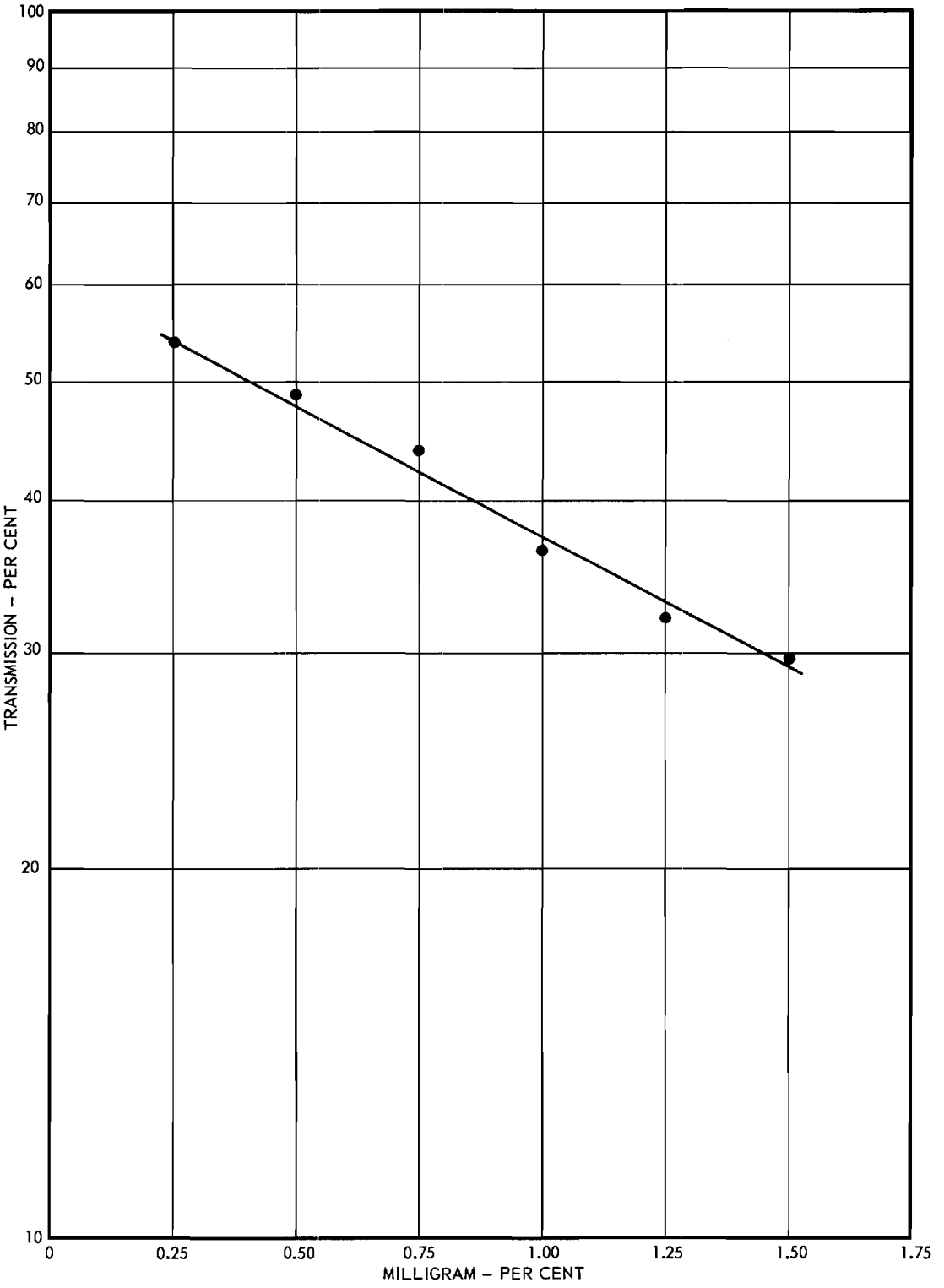

Figure 4. Per Cent Transmission Values of Different Concentrations of Serratia marcescens, ATCC 274, at 7.2 Microns Wavelength.

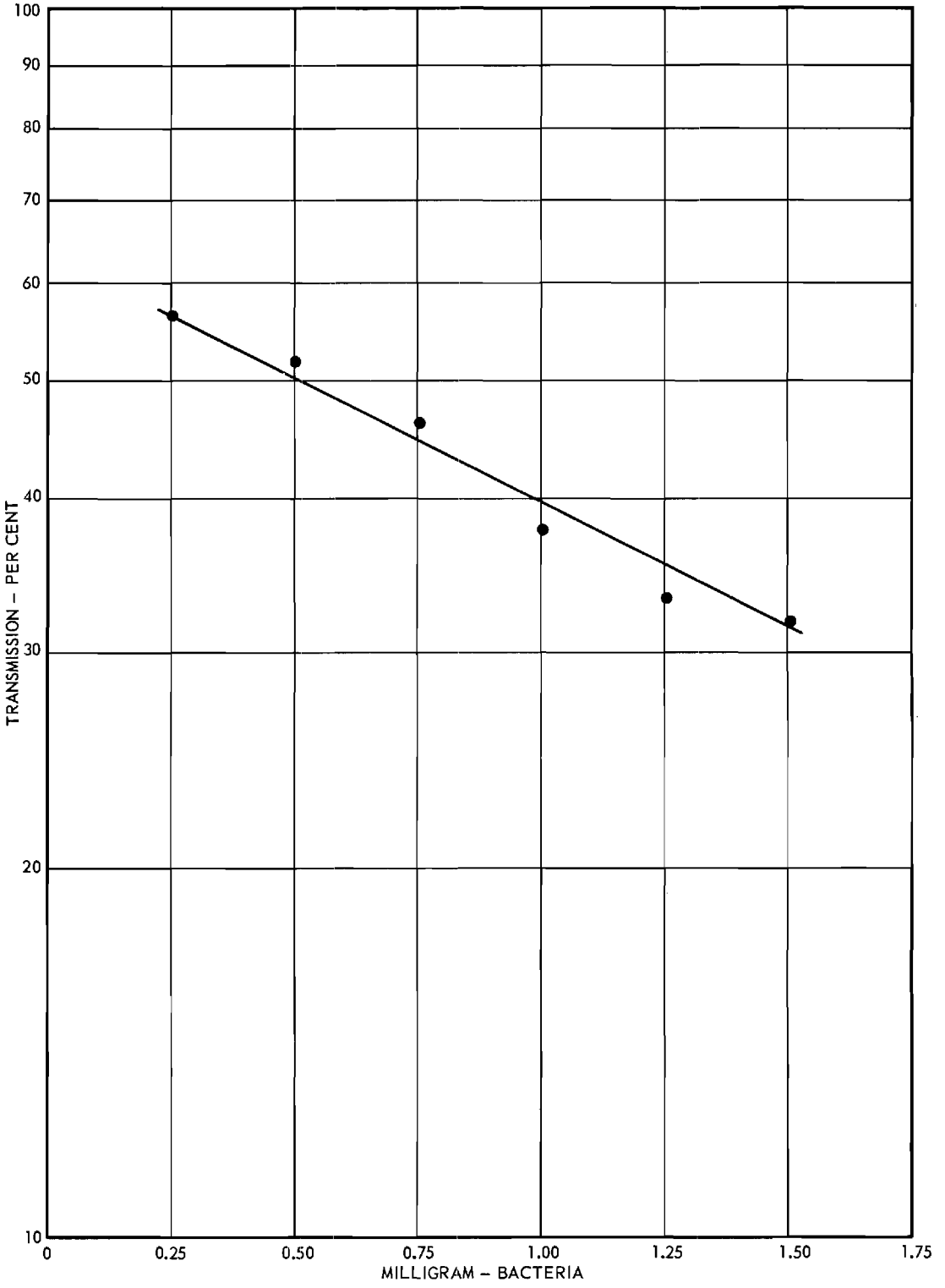

Figure 5. Per Cent Transmission Values of Different Concentrations of Serratia marcescens, ATCC 274, at 8.1 Microns Wavelength.

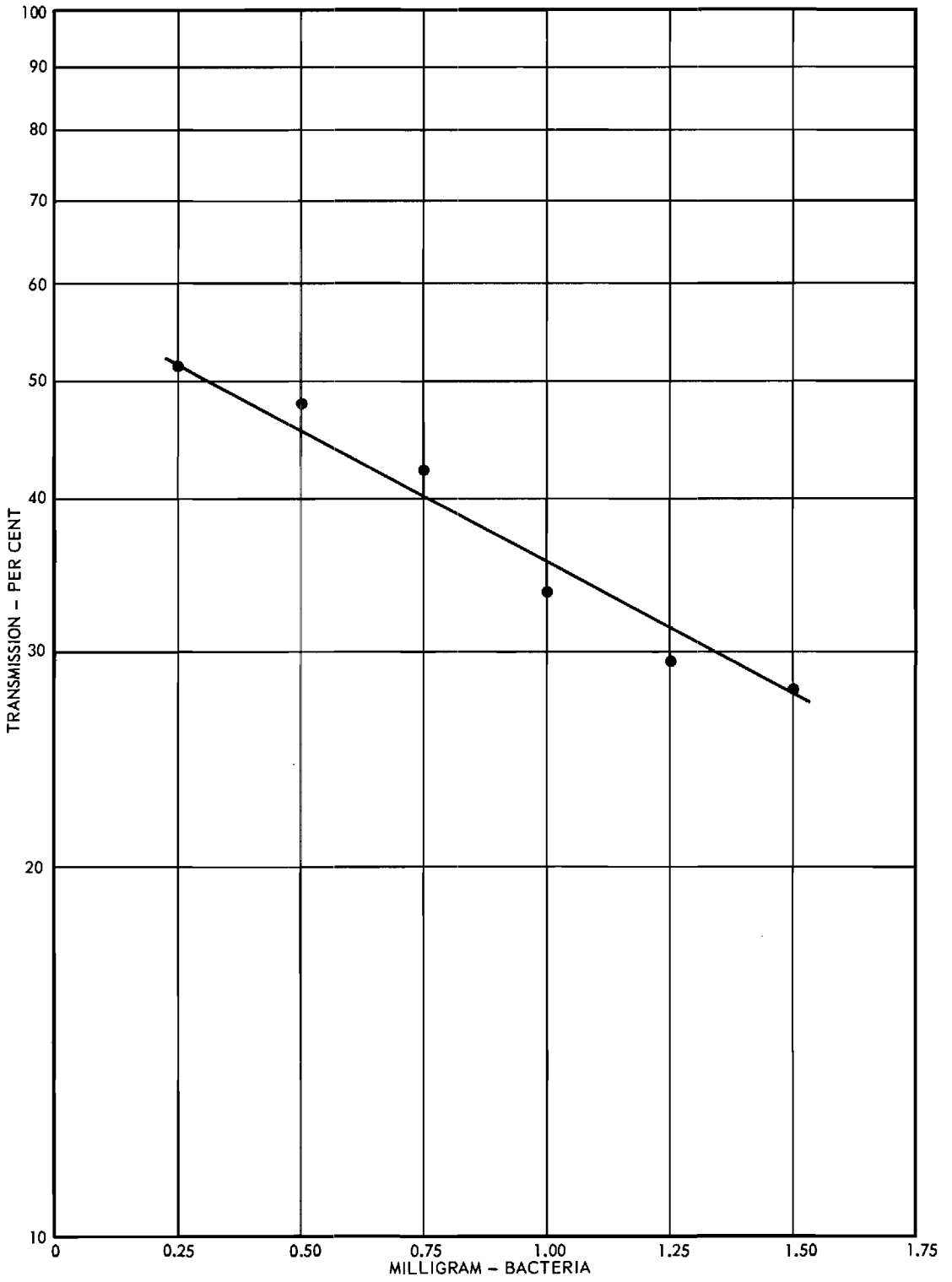

Figure 6. Per Cent Transmission Values of Different Concentrations of Serratia marcescens, ATCC 274, at 9.35 Microns Wavelength.

TABLE III

PER CENT DEVIATION FROM THEORETICAL -- LAMBERT ABSORPTION LAW

Bacterial Concentration	Wavelength in Microns					KBr-Bacteria Disc	
	6.53	6.9	7.2	8.1	9.35	Weight	Thickness
(Mg)	(%)	(%)	(%)	(%)	(%)	(Mg)	(Mm)
0.25	+0.5	-1.0	0	0	0	288.91	0.787
0.50	+1.5	+1.0	+1.0	+1.5	+2.5	292.35	0.800
0.75	+1.5	+1.0	+1.5	+1.0	+2.5	292.18	0.807
1.00	-3.0	-1.2	-1.0	-2.0	-2.0	289.23	0.800
1.25	-1.5	-1.5	-1.0	-2,0	-2.0	292.91	0.810
1.50	+0.5	+0.5	+0.5	0	0	295.47	0.807
Average per cent deviation	-0.08 ±1.8	-0.20 ±1.2	+0.16 ±1.02	-0.25 ±1.46	+0.16 ±2.0	291.84 ±2.45	0.801 ±0.018

Losses of material during handling of the potassium bromide - bacterial mixture preparatory to forming the pressed disc appear to be of the order of 3 per cent by weight. The average weight of the finished disc is 291.84 ± 2.45 milligrams. The average thickness of the disc is 0.801 ± 0.018 millimeters.

Reproducibility of the instrument settings and the sustained quality of the stored disc was of prime interest. Frequently there was an interval of several days between preparation of the discs and their spectroscopic examination. Two such discs of variants BR and W were examined initially and again after the lapse of 30 days. The results are shown in Figure 7. Peaks of transmission and absorption are in agreement with 0.5- to 1.5-per-cent variation. This variation is exceeded at one absorption point (6.05 microns, W-variant) where the disparity of the replicate spectra is 2 per cent.

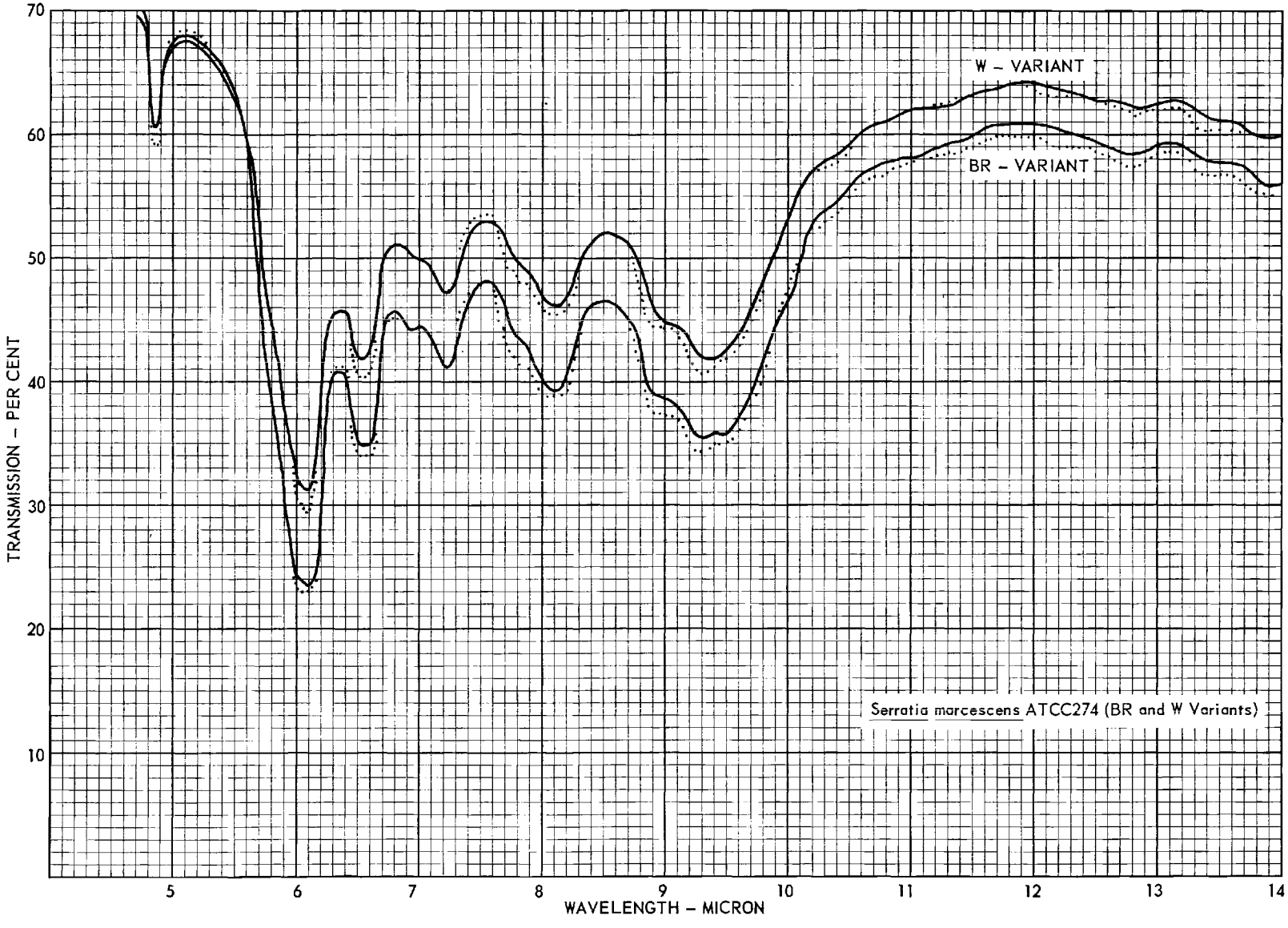

Figure 7. Spectrum Reproducibility and Storage Stability of Prepared Sample.

2. Relationship Between Results of Infrared Spectroscopy and Aerial Viability

a. Variants of Serratia marcescens, ATCC 274. The procedure of transforming the per cent transmission values to wavelength units[10] was employed to examine the spectra obtained. The basis of this transformation is the long wavelength side of the carbohydrate band (9.2 to 10.2 microns). Projections of the maxima and minima bands are extended to this line, and from the intersection of these two lines a vertical line is drawn to the wavelength scale. The wavelengths of these perpendicular lines are determined to the nearest 0.01 micron and recorded as a three digit number (centimicrons). These units are independent of sample thickness and scale differences.[10] Random inspection of the spectra of the chromogenic variants shows that the assigned value of ±2-per-cent variation in transmission causes as much as a ±7 centimicron variation. Upon this basis the spectroscopic results shown in Figures 8 to 11 do not indicate a separation of the chromogenic variants of Serratia marcescens ATCC 274 when compared on the centimicron scale at selected wavelengths (Figure 12). The wider separation of the R-variant at the wavelengths of 6.35 and 6.55 microns are considered as exceptional and not a reflection of a real spectral difference. Further, attempts to assign the k values to some rank order of the spectral characteristics has been without success. The constant relative position of the R-variant in the centimicron scale was considered to have some possible significance but the evidence to support any conclusions is lacking. Of interest, but apparently not relatable to k correlation, is the higher actual transmission values of the W-variant.

In an attempt to possibly amplify any qualitative differences which might exist, spectra were obtained of the BR-variant and the W-variant at 18, 24, and

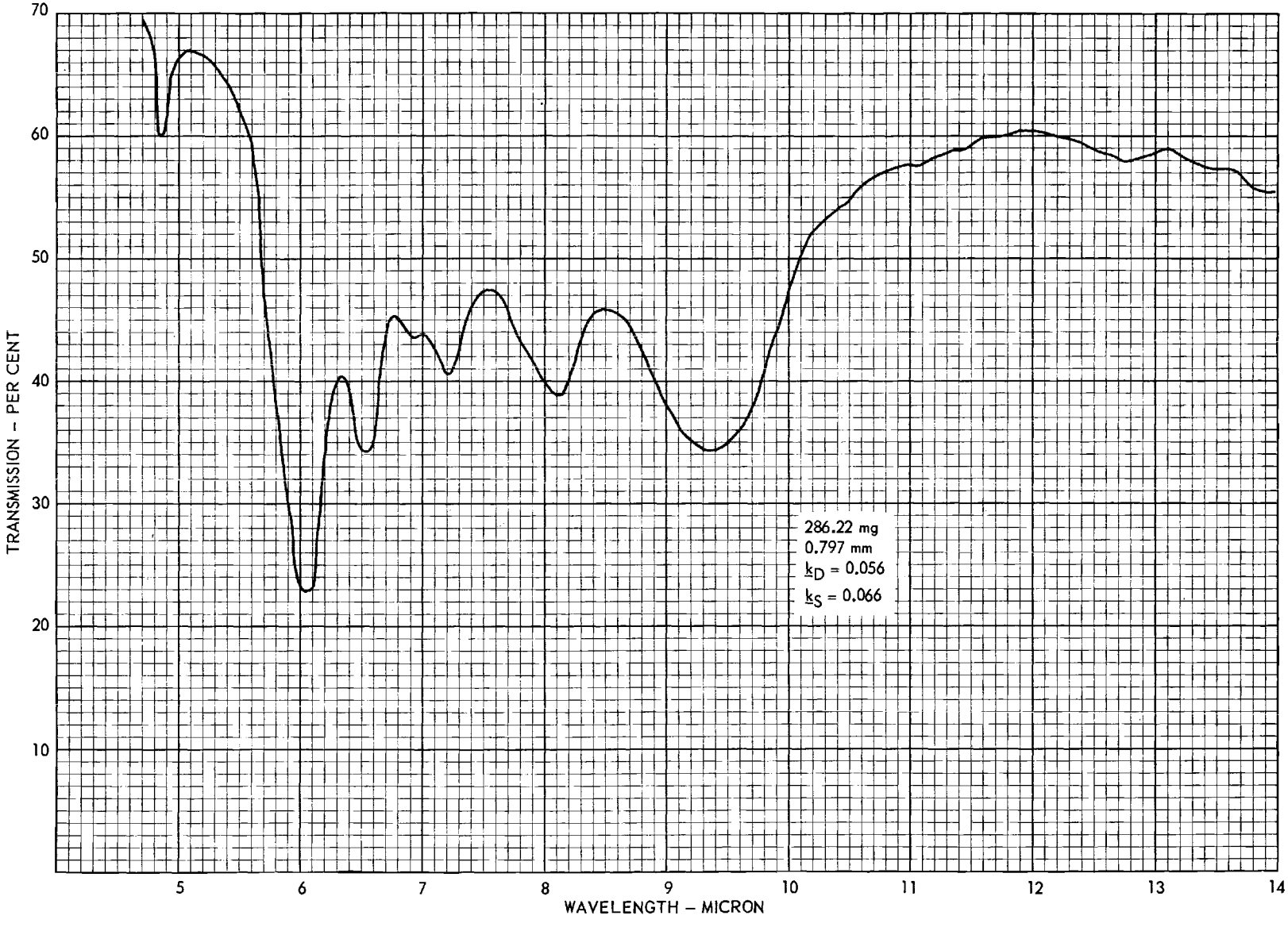

Figure 8. Spectrum of Blood-Red Variant (Serratia marcescens, ATCC 274).

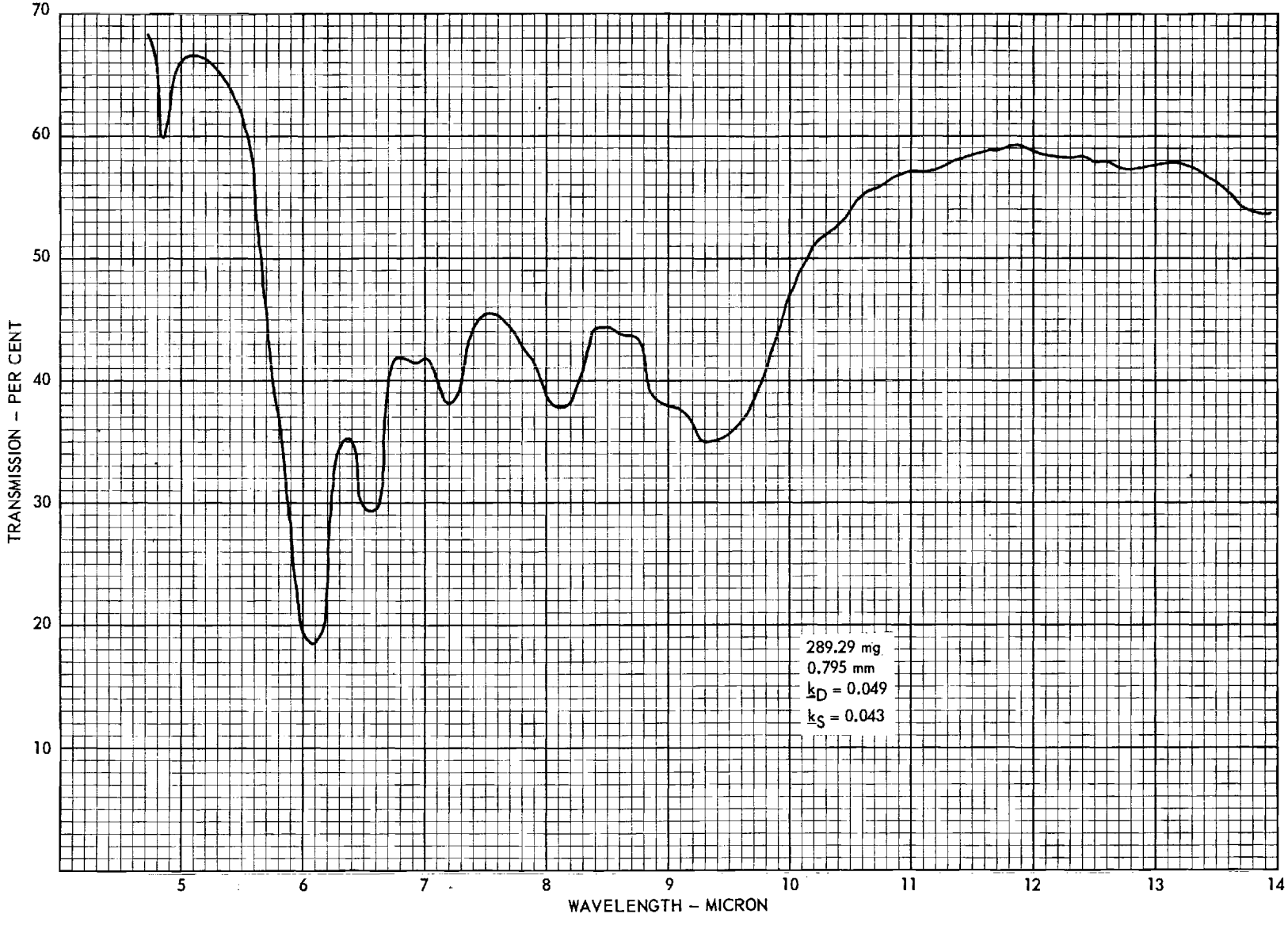

Figure 9. Spectrum of Red Variant (Serratia marcescens, ATCC 274).

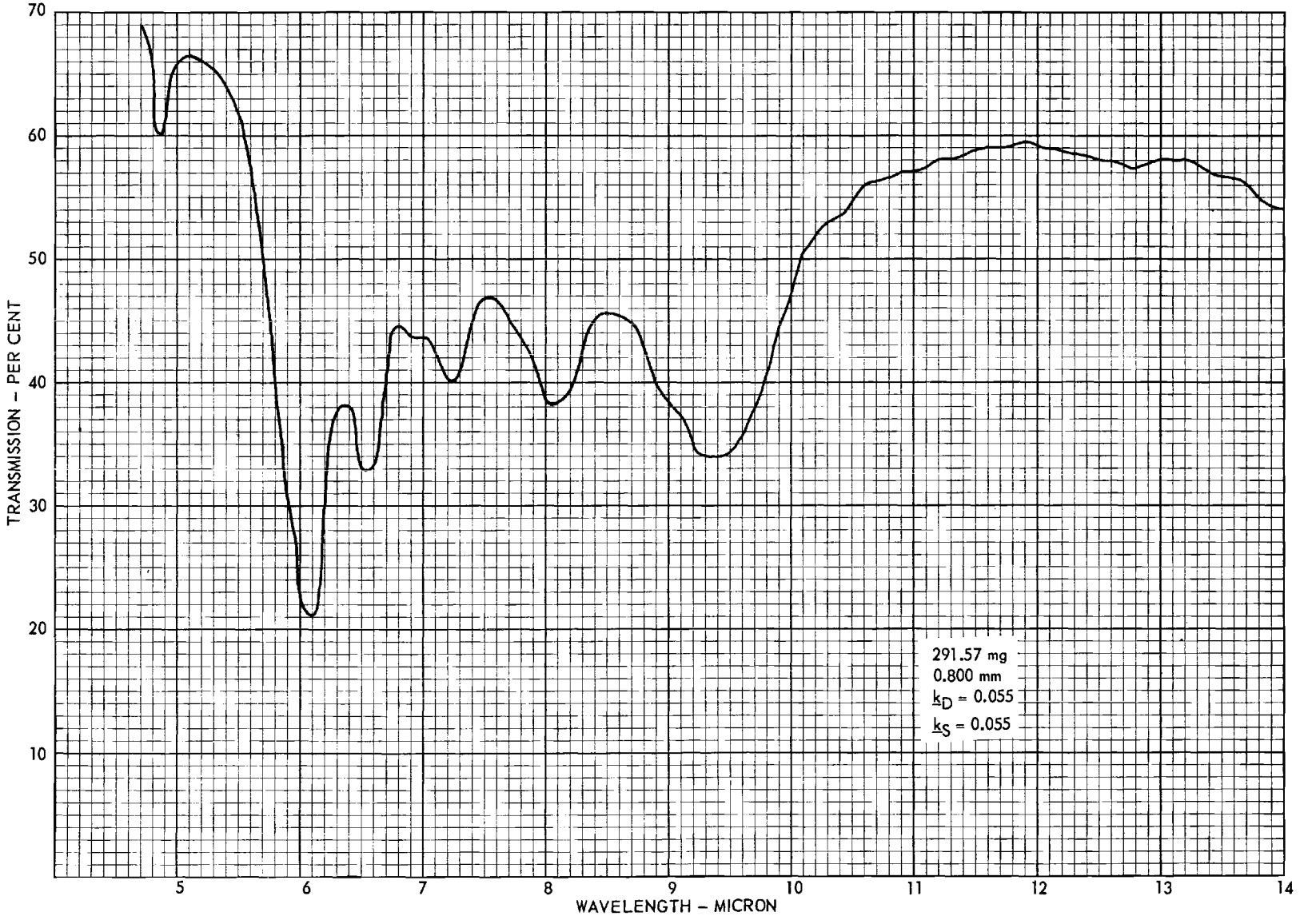

Figure 10. Spectrum of Pink Variant (Serratia marcescens, ATCC 274).

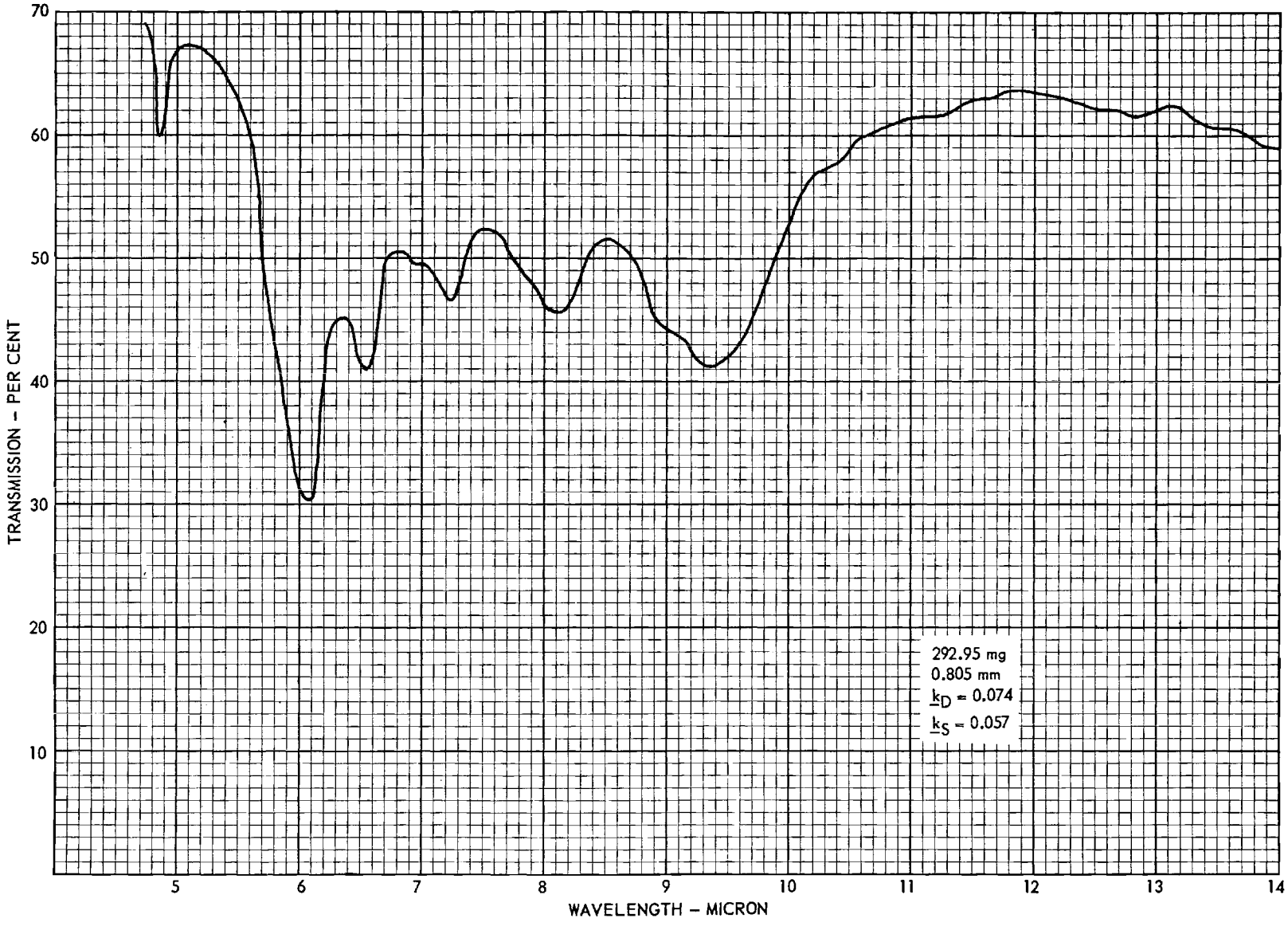

Figure 11. Spectrum of White Variant (Serratia marcescens, ATCC 274).

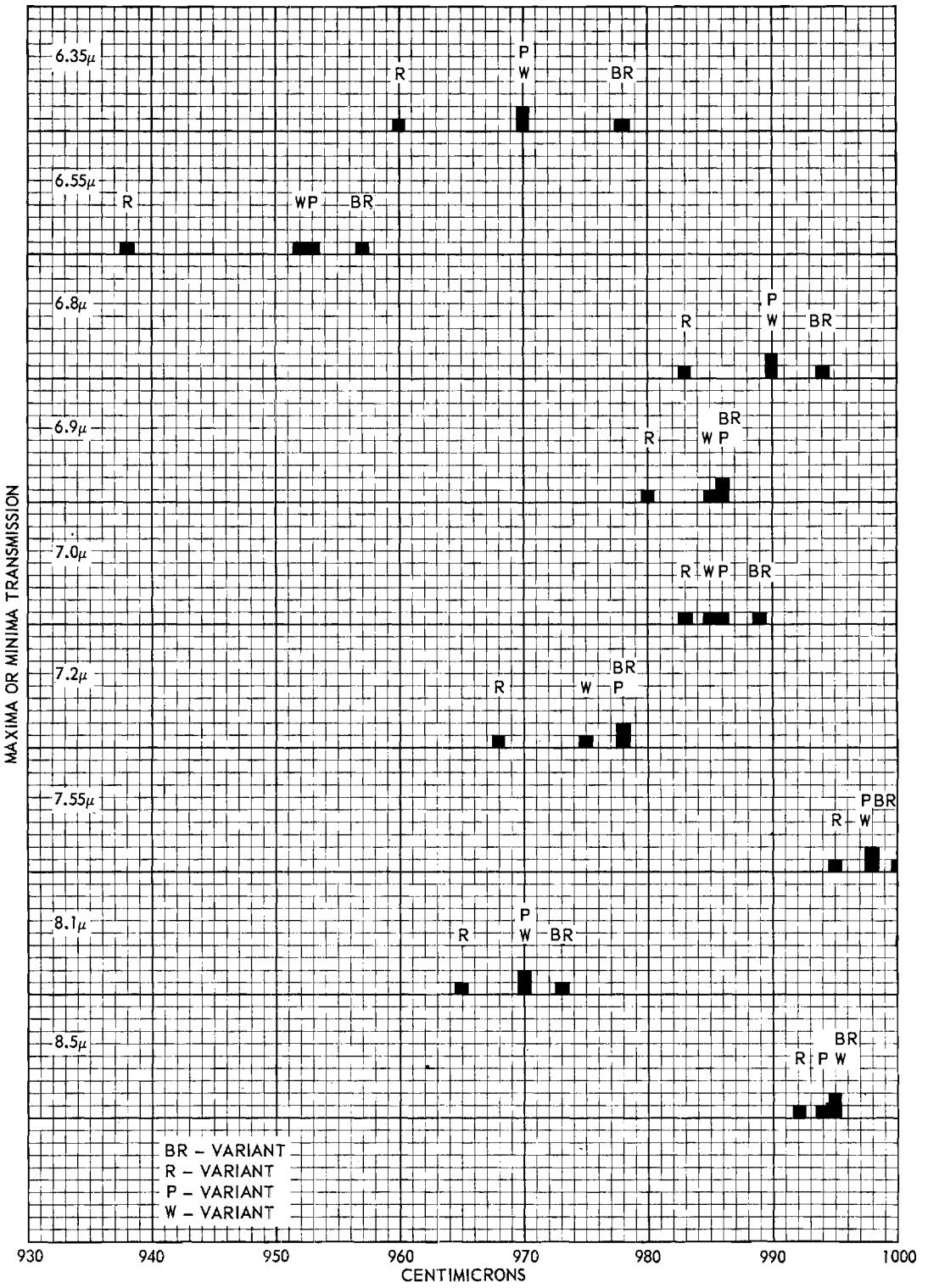

Figure 12. Comparison of Variants of Serratia marcescens, ATCC 274, on Centimicron Scale.

30 hours culture age. The results did not add anything to the information already gained.

b. Species of Serratia and Escherichia. The same examination of spectra of species of Serratia and Escherichia was made with the transformation of transmission values to the centimicron scale. Figures 13 to 20 show the spectra of the species of these two genera. In Figure 21 is the summary comparison of the species of these two genera on the centimicron scale. The differentiation possible among the species and genera is considerably enhanced at this level of classification. However, the primary objective is not so much the identification of these organisms but their examination for a possible relationship of their spectra to their aerial viability. This relationship may or may not bear any sort of classification contingency. In seeking a possible pattern relationship among this particular group, the static k values were selected for the reason of their more sensitive reflection of reduced aerial viability. There appears to be an approximate grouping of organisms having the same general k value. This grouping seems to hold reasonably well at the wavelengths of 6.8, 6.9, 7.0, 7.2, and, to a lesser extent, 7.55 microns. This approximate grouping is rendered less acceptable by the occurrence of Serratia marcescens ATCC 274 (k 0.046) with Escherichia freundi ATCC 8454 (k 0.12) and Escherichia coli ATCC 10536 (k 0.091). The preliminary nature of the data does not warrant more than the indication that this grouping might have a basis in fact.

A grouping of the spectra of the genera Serratia and Escherichia in order of decreasing total transmission (Figure 22) to determine if any qualitative differences might be existent does not show any rank-order relationship with k. These spectra are arranged at 3-per-cent differences based on the internal

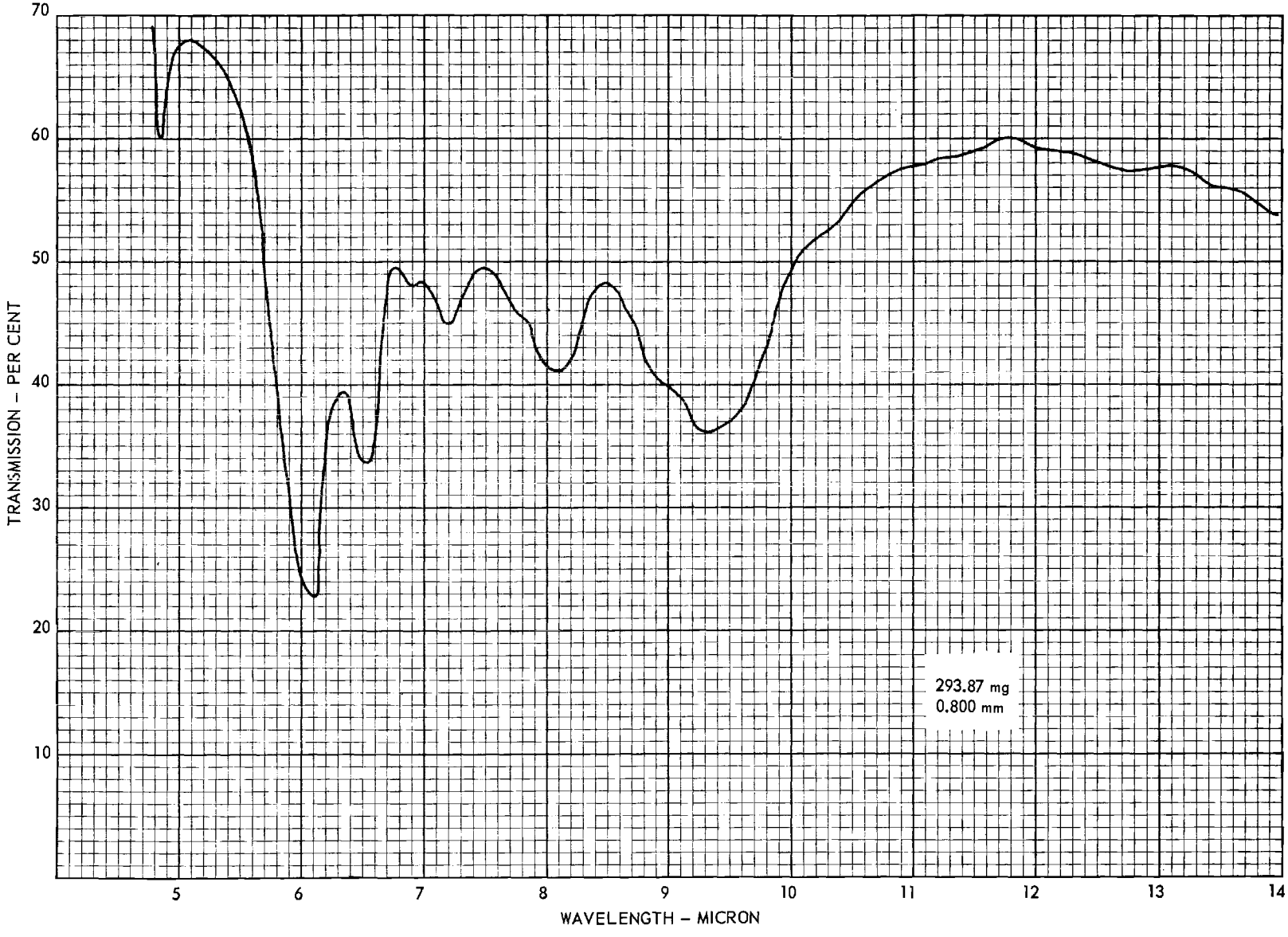

Figure 13. Spectrum of Serratia plymuthicum, ATCC 183.

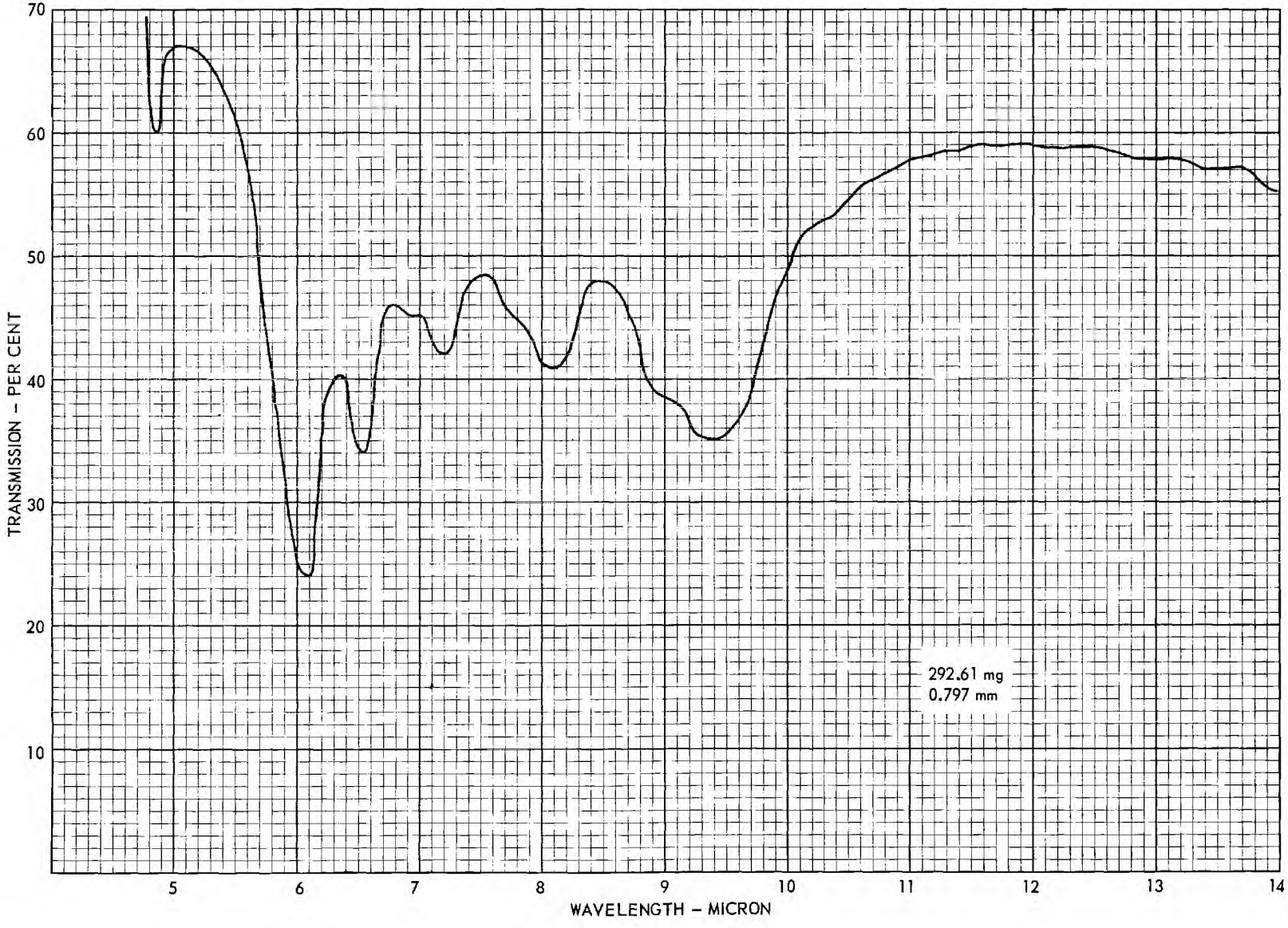

Figure 14. Spectrum of Serratia anolium, ATCC 6065.

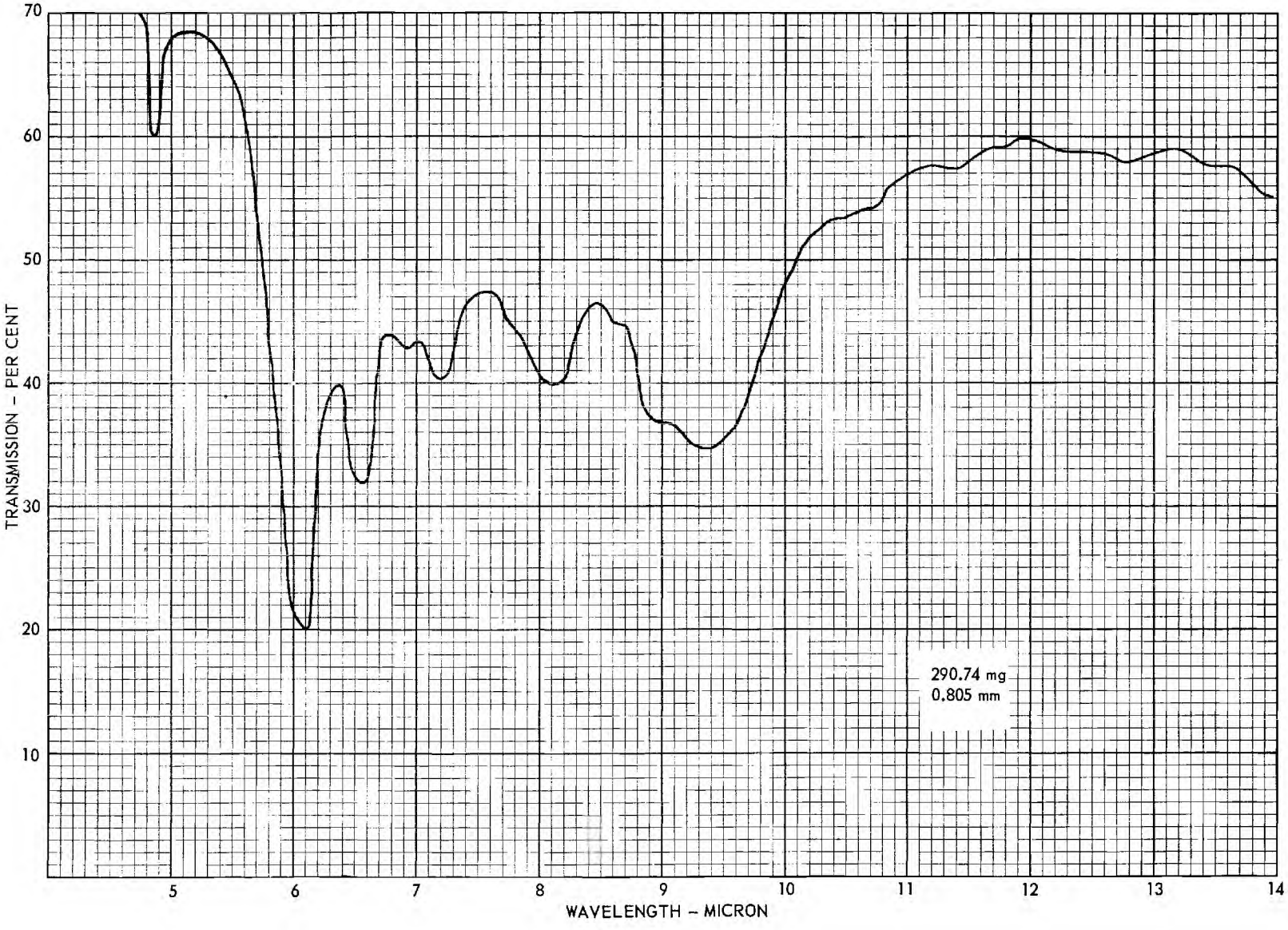

Figure 15. Spectrum of Serratia indica, ATCC 4003.

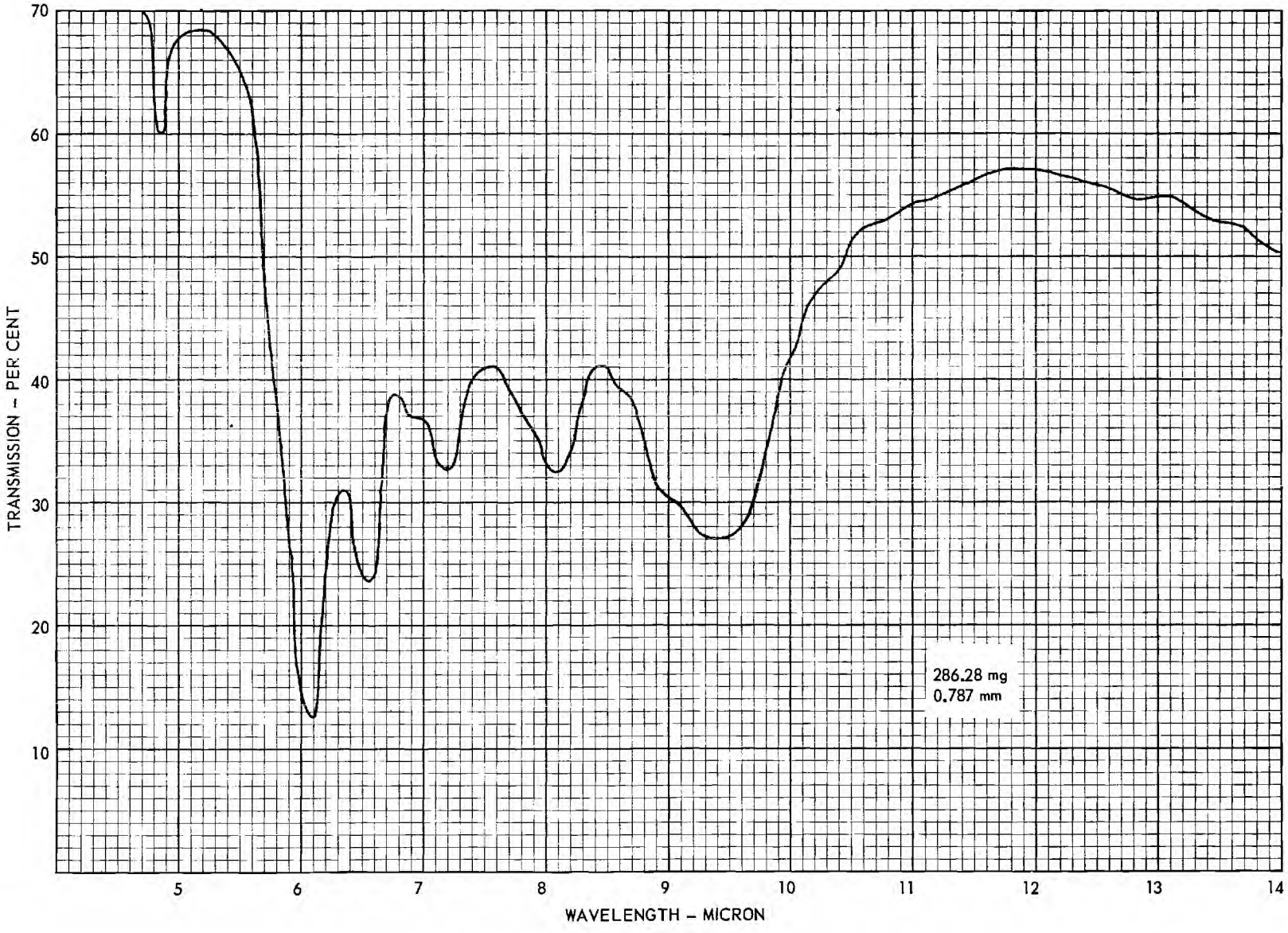

Figure 16. Spectrum of Serratia urinae, ATCC 11111.

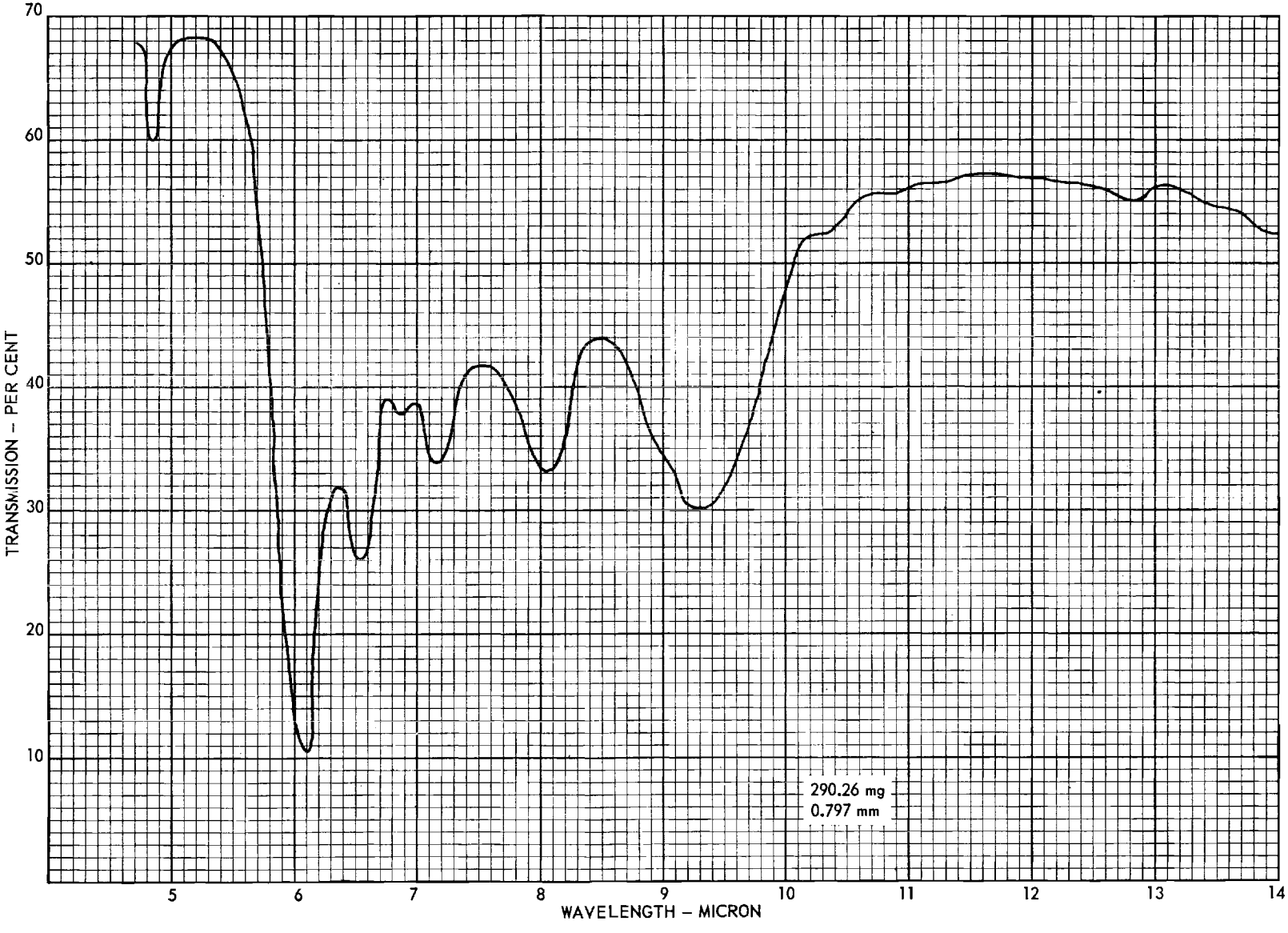

Figure 17. Spectrum of Serratia marcescens, ATCC 274.

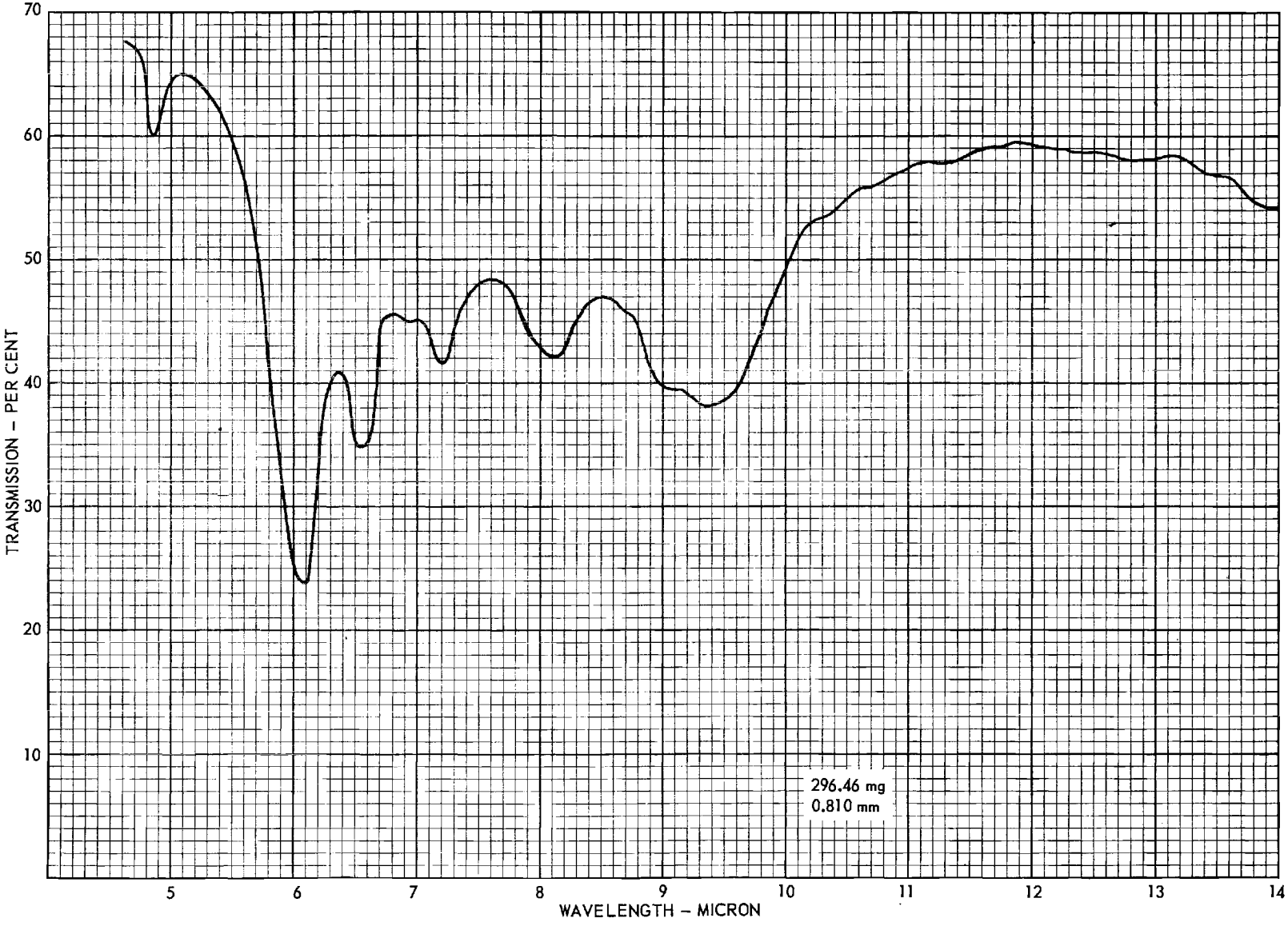

Figure 18. Spectrum of Escherichia intermedium, ATCC 6750.

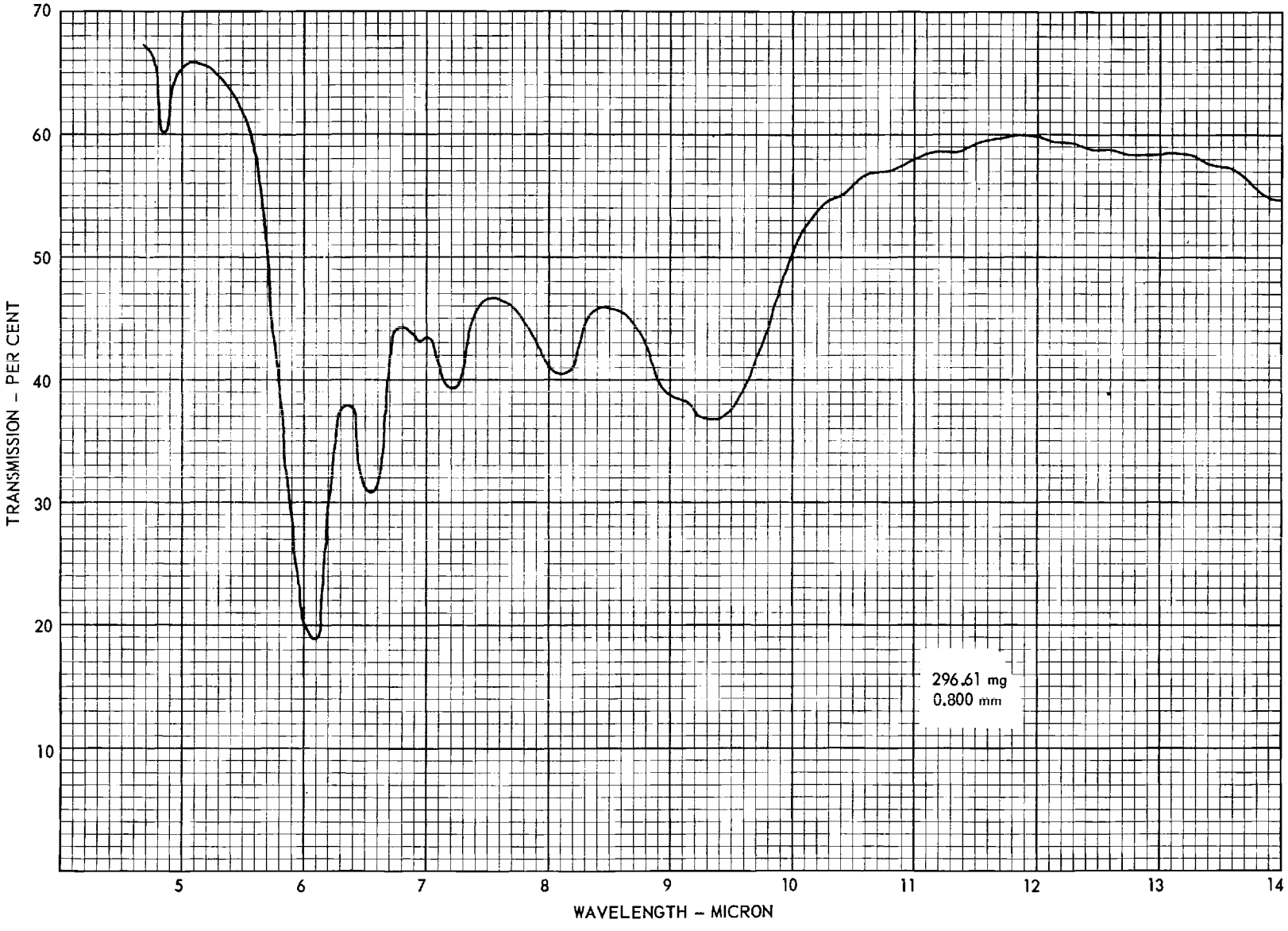

Figure 19. Spectrum of Escherichia freundi, ATCC 8454.

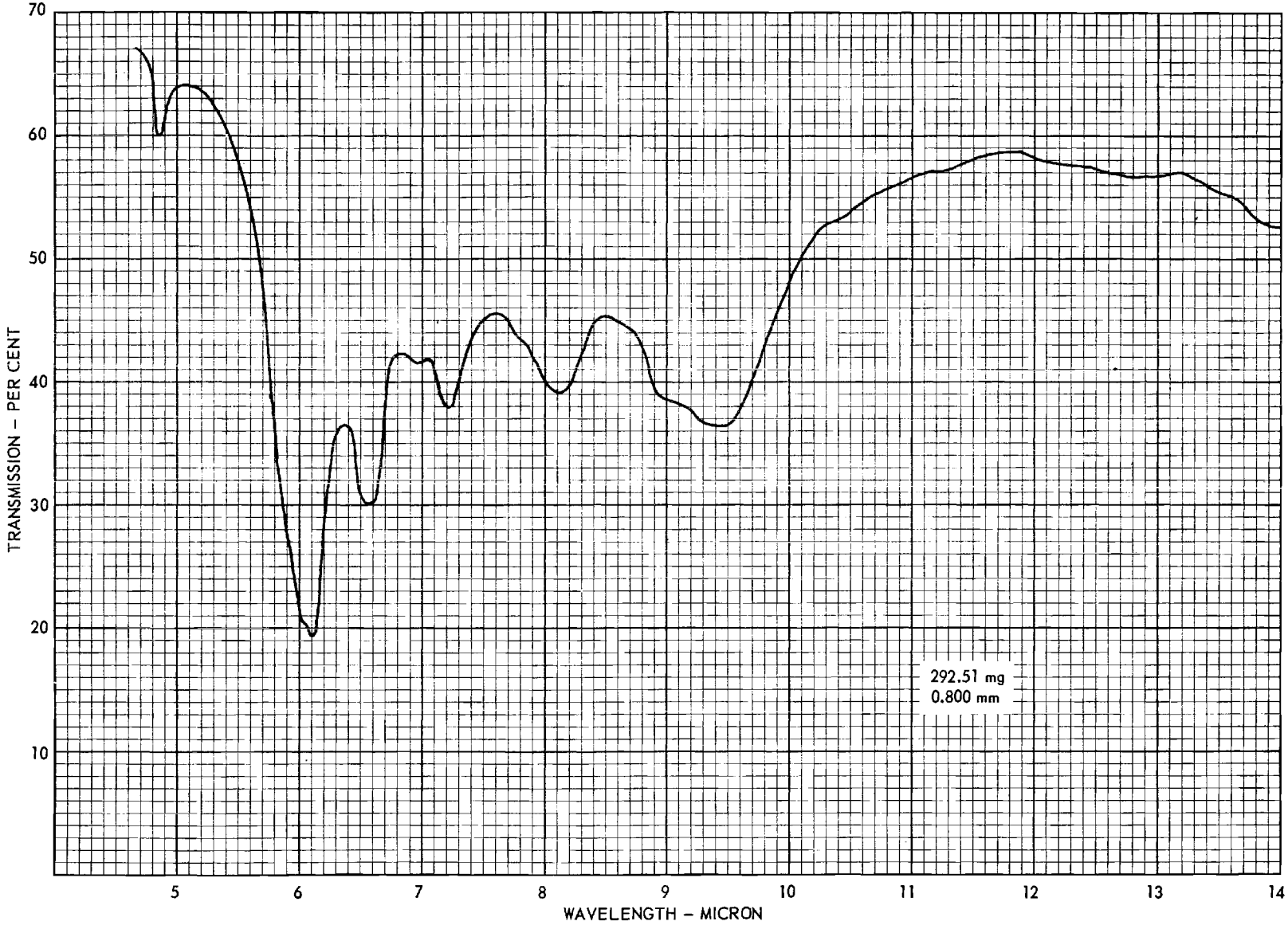

Figure 20. Spectrum of Escherichia coli, ATCC 10536.

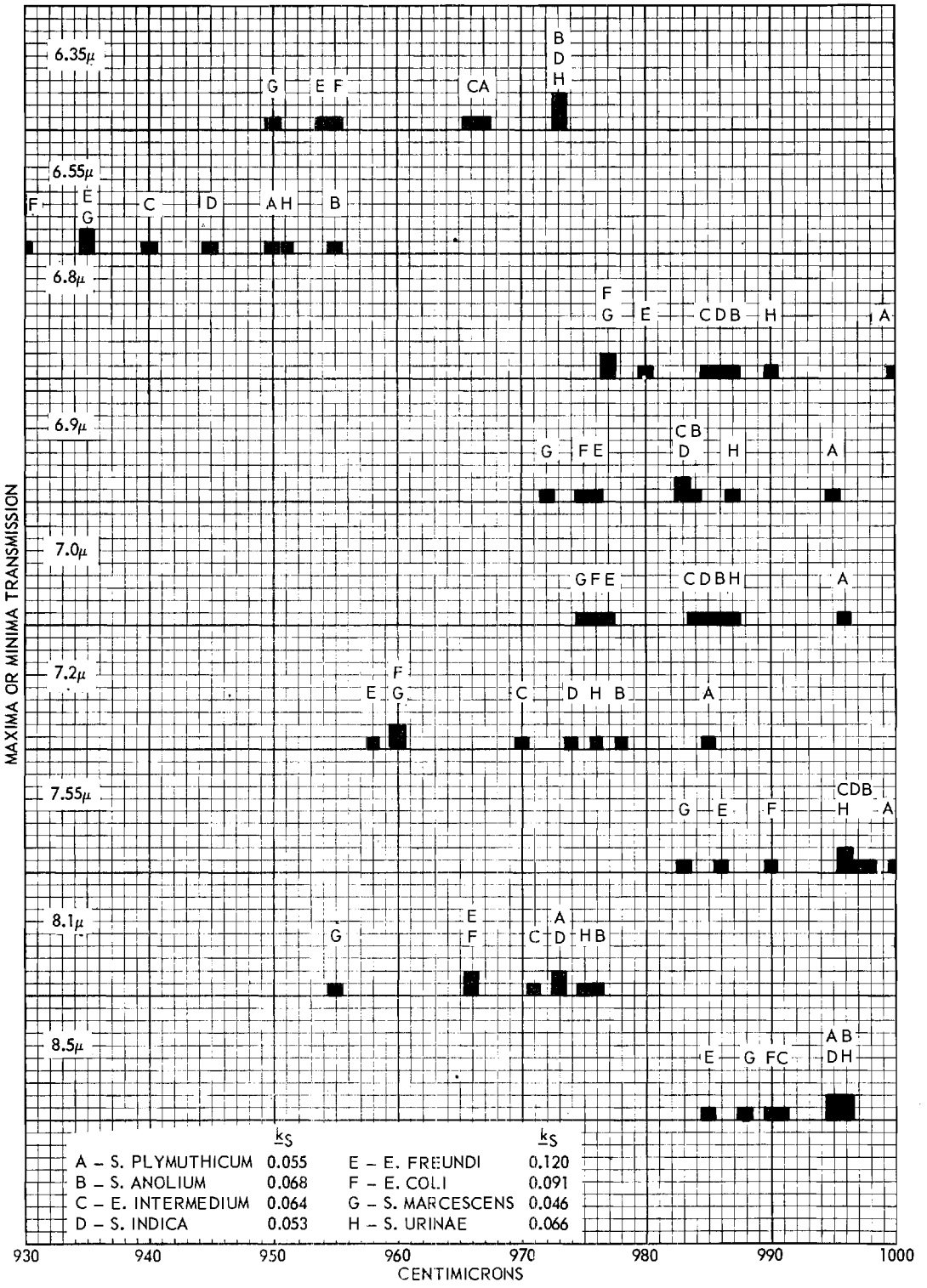

Figure 21. Comparison of Species of Serratia and Escherichia on Centimicron Scale.

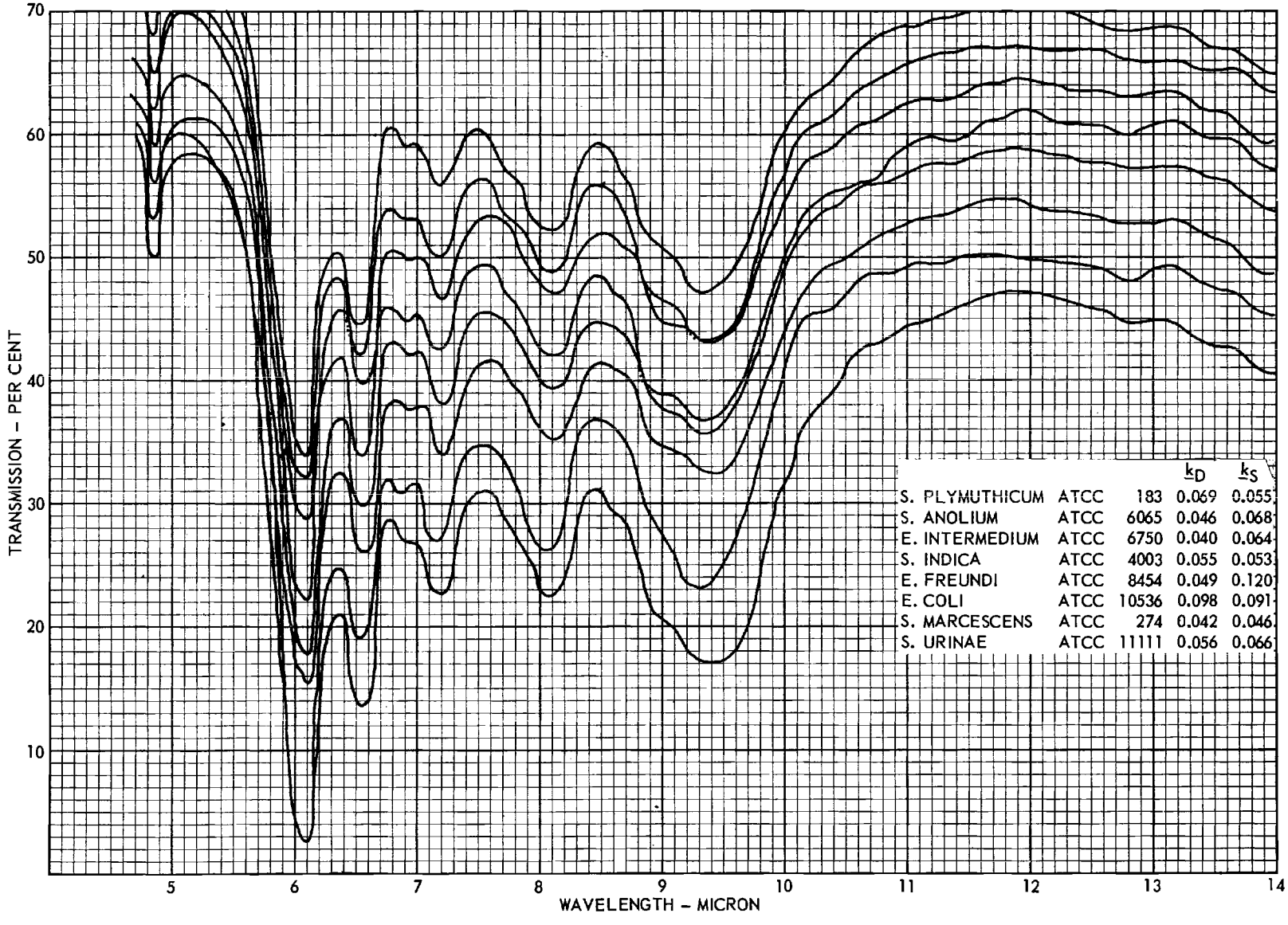

Figure 22. Spectra of Serratia and Escherichia Species Ranked in Order of Decreasing Transmission.

standard of potassium thiocyanate. It is of interest to note that ratio of per cent transmission at 7.2 microns and 8.1 microns (7.2:8.1) is less than unity for the genus Escherichia and more than unity for the genus Serratia.

VI. DISCUSSION

The pressed potassium bromide disc technique has been applied to quantitative analysis[11] as well as to the examination of biological materials.[12] There are several features of this technique which commend its use. Among them are the absence of scattering losses, no obscuring of the spectral region by the suspending medium, better separation and sharpness of bands, simplification of quantitative work, and facility of sample storage for future reference. Further, in circumstances of irregularly scheduled usage of the infrared spectrophotometer, it is expedient to have readily available prepared samples which are stable in storage.

The influence of particle size distribution of the potassium bromide is of significance in the application of this procedure for quantitative work. Spectral quality of the combined materials may be increased by grinding in the presence of a volatile solvent,[13] which in this application was diethyl ether. The addition of volatile solvent not only improved the optical qualities of the disc but aided greatly in the homogenous dispersion of the internal standard.[14] The incorporation of an internal standard serves the dual function of providing an accurate wavelength marker and providing a measure of the reproducibility of each disc. The performance of the potassium bromide technique during the course of this exploratory study appears to justify its selection for this particular application.

Previously published studies on the application of infrared spectroscopy to the identification of bacteria[2,3,4] emphasize the vital importance of duplication of instrument performance. The initial failure to resolve a bacterial differentiation always has been shown to be due to a deterioration in the

performance of the spectrophotometer.[2] The spectrophotometer used in this work is employed in daily routine operation in the Department of Chemistry where it is in more or less continual operation by a number of persons. Experience with this instrument has served to emphasize the crucial requirement of instrument performance in elucidating the sometimes very small differences between bacterial species. It is felt that the prime source of error involved in this study resides in the instrument performance. The variation of ±2 per cent encountered is excessive, being an error greater than the magnitude of differences existing between some bacterial species. A variation no greater than ± 1 per cent would be most desirable, although very good identification made at ± 1-1/2-per-cent variation has been reported.[3] It has been suggested[4] that for a systematic study of bacteria a grating instrument with the same resolving power as a prism instrument and utilizing wider slit widths be developed for that purpose.

It has been reported that it is possible to distinguish bacterial strains, but the spectral differences presently attainable are of the same order of magnitude as those arising from the yet uncontrolled errors of the technique employed.[4] Some small variation in spectral reproducibility of a species has been attributed to different strains, some species showing more variation due to strain effect.[3] The separation of Serratia marcescens ATCC 274 into several components based upon chromogenicity is a further dichotomy in the classification schema and the attempts to characterize these components by their infrared spectra extends the utility of this spectroscopic method beyond its present capabilities. The inability to distinguish these chromogenic fractions having variable response to the airborne state obviates the use of the spectroscopic method and indicates continued reliance upon actual aerial tests to monitor the standard test organism.

An additional approach to the problem might be the selection of certain culture media which might reflect a differential utilization by the chromogenic variants, a metabolic variation demonstrable by infrared spectrographic analysis. The feasibility of this general approach is indicated at the species level by easier differentiation of cultures grown on a carbohydrate medium, which was unequally utilized by the species examined.[15] This approach might be extensible to subspecies or strain level.

The suggested indication that there may be some degree of association between the aerial viability (k) and infrared spectral grouping might be of interest to pursue further. Any study of this type would lean heavily on a statistical basis and would consequently involve a considerable number of replicate determinations.

Respectfully submitted:

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Approved:

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J. E. Boyd, Director
Engineering Experiment Station

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